12-2019

Xenobiotic Exposure Requires Mitochondrial Metabolism for REDOX Homeostasis and Survival in Astrocytes

Jordan Rose
University of Nebraska-Lincoln, jomiro3777@gmail.com

XENOBIOTIC EXPOSURE REQUIRES MITOCHONDRIAL METABOLISM FOR REDOX HOMEOSTASIS AND SURVIVAL IN ASTROCYTES

by

Jordan Rose

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillments of Requirements

For the Degree of Master of Science

Major: Biochemistry

Under the Supervision of Professor Rodrigo Franco-Cruz

Lincoln, Nebraska

December, 2019

XENOBIOTIC EXPOSURE REQUIRES MITOCHONDRIAL METABOLISM FOR REDOX HOMEOSTASIS AND SURVIVAL IN ASTROCYTES

Jordan Rose, M.S.

University of Nebraska, 2019

Advisor: Rodrigo Franco-Cruz

Astrocytes are integral components of glutamatergic neurotransmission, providing essential metabolic processes for neuronal homeostasis and acting as the first line of defense against xenobiotics crossing the blood brain barrier. Arsenic is a xenobiotic with widespread natural and industrial prevalence, and has been linked to impaired neurodevelopment and neuronal death. Given the integrated metabolic nature of astrocytes and neurons, we sought to explore how arsenic impacts astrocyte metabolism in order to better understand the mechanisms of xenobiotic toxicity in the mammalian brain.

We demonstrate that astrocyte viability depends upon *de novo* glutathione (GSH) synthesis during arsenic exposure, and sub-lethal arsenic exposure (iAs <10μM) increased intracellular GSH. Multidrug resistance-associated protein 1 (MRP1) inhibition increased intracellular arsenic accumulation and sensitized the astrocytes to arsenic. A 24hr time course of intracellular arsenic accumulation indicated that arsenic efflux is achieved 3hrs following exposure, demonstrating naive and adaptive phases.

U-13C-glucose NMR-based metabolomics revealed that iAs induced an anaplerotic generation of glutamate via the tricarboxylic acid cycle and its extracellular release in astrocytes. Quantification of the extracellular glutamate indicated potentially

excitotoxic concentrations were reached (Glu > 20μM), which were not significantly altered by γ-glutamyl transpeptidase (γGT) or MRP1 inhibition. Excitatory amino acid transporter 1 and 2 (EAAT1/2) inhibition significantly increased extracellular glutamate accumulation even in the absence of arsenic.

Mitotoxins rotenone (Rote), paraquat (PQ), or 1-methyl-4-phenylpyridinium (MPP^+) sensitized the astrocytes to arsenic. Inhibition of mitochondrial carbon inputs by etomoxir (Eto) or UK5099 (UK), or of transamination by aminooxyacetic acid (AOAA), sensitized astrocytes to arsenic to a similar degree. Eto, UK, and AOAA increased arsenic accumulation in astrocytes but did not significantly alter extracellular glutamate accumulation.

This thesis illustrates that mitochondrial metabolism is essential to astrocytes survival during xenobiotic exposure. Further, the significant efflux of glutamate may create toxic conditions for neurons. Astrocytes previously have been shown to be glycolytic, resistant to mitochondrial toxins, and viable *in vivo* without functional electron transport chain. These results now demonstrate that mitochondria metabolism is essential for astrocytes survival under conditions of stress and require us to reassess the role of mitochondrial metabolism in astrocytes.

TABLE OF CONTENTS

List of Figures

Chapter 2

Chapter 3

Chapter 4

APPENDIX A

CHAPTER 1

INTRODUCTION AND BACKGROUND INFORMATION

Xenobiotics are substances, compounds, or elements that are found at concentrations in an organism that are not expected to be there. The term xenobiotic is derived from the Greek words xeno (foreign) and bios (life), and is often used as an encompassing term for anything toxic to an organism. It should be noted however that xenobiotics are context specific: while the mitochondrial toxin rotenone is considered a xenobiotic in a mammalian system, it is not considered a xenobiotic when found within legumes that produce it unless the concentration of rotenone greatly exceeds the expected concentrations found within that specie. In humans, environmental xenobiotic exposure has been linked to a variety of neurodegenerative diseases and irregular neurodevelopment, such as Parkinson's Disease and Alzheimer's Disease (see (Bondy, 2016) and (Rock and Patisaul, 2018) for more details). As xenobiotic toxicants are prevalent in our environment, it is important to understand their effects in order to better treat the symptoms of their toxicity, particularly when the effects of chronic exposure are not readily observable.

Arsenic is a group 15 metalloid element that is considered a xenobiotic to the mammalian system and has a widespread and varied natural distribution within the Earth's crust. Arsenic is also found in many industrial settings, including waste products from metal smelting, as an n-type dopant in semiconductors, as a component of alloys and batteries, and as a wood preservative. Additionally, the toxic properties of arsenic have been exploited in herbicides, pesticides, and leukemia treatment. Arsenic exposure in humans primarily occurs through ingestion of arsenic contaminated water or food, and inhalation of arsenic dust, particularly in industrial settings. Arsenic is highly water soluble, and groundwater sources can be highly contaminated from natural, industrial, and/or agricultural sources (Nordstrom, 2002;Duker et al., 2005). The danger

that can occur from arsenic contaminated water was highlighted during the 1980s in Bangladesh, where ground water wells created to provide clean water to the population were years later found to contain high concentrations of arsenic (for an in depth review, see (Chakraborti et al., 2015)). At the time of assessment, it was estimated that 21 million people had been exposed to water with arsenic above 50 µg/L (the maximum permissible limit by the World Health Organization (WHO)), with twice as many above 10 µg/L (the recommended WHO limit, and the limit adopted by the United States in 2001) (Smith et al., 2000). In 2011, an approximate 43,000 deaths/year in Bangladesh (5.6% of all deaths) were attributable to arsenic related poisoning and cancers (Sohel et al., 2009;Flanagan et al., 2012), highlighting the danger of arsenic contamination. Further, in 2010 an estimated 2.1 million Americans are consuming water from wells above the 10 µg/L limit of the Environmental Protection Agency (EPA).

Arsenic exposure has been found to negatively impact neurodevelopment. Multiple inverse correlations have been found between intelligence quotient (IQ) parameters and environmental arsenic exposure during childhood (Rosado et al., 2007;Dong and Su, 2009;Hamadani et al., 2011;Wasserman et al., 2014). Many studies have focused on the effects of arsenic on neurons, demonstrating neuronal cell death, dysfunction, and malformation during arsenic exposure in development (Namgung and Xia, 2001;Chattopadhyay et al., 2002a;Chattopadhyay et al., 2002b;Wang et al., 2010;Kaler et al., 2013). The mitochondria of neurons are particularly affected by arsenic exposure, which is a prominent source of neuronal cell death given neuronal dependence upon oxidative phosphorylation (see (Prakash et al., 2016) for a review, as well as chapters 2 and 3). While the effects of arsenic on neurons have been extensively studied, far less research has been directed towards the effects of arsenic on the astrocytes. As reviewed in more detail in chapters 2 and 3, astrocytes envelope the brain capillaries with their processes, making them the first cell type to contact toxins capable of passing the blood brain barrier, such as arsenic. Further, astrocytes have a cooperative

metabolism with neurons, maintain neuronal REDOX homeostasis, neurotransmitter homeostasis and clearance, and provide neurons with bioenergetic balance with lactate (see chapter 3). A key aspect of astrocytes is that they are glycolytic, and do not depend on oxidative phosphorylation (Supplie et al., 2017). This makes astrocytes far more resistant to mitochondrial poisons compared to their neuronal counterparts (Bolanos et al., 1995;Almeida et al., 2001), and has led to the astrocyte mitochondria in particular being understudied in xenobiotic toxicity.

Because neurons and astrocytes are highly interconnected cell types, and disruption of astrocyte functions can impact neurons and neurotransmission, it is important to understand how a xenobiotic, such as arsenic, impacts the functions of astrocytes to understand how xenobiotics affect brain functions. This thesis will begin with a review of central nervous system cell types and signaling, transition into astrocyte metabolic functions with an emphasis on mitochondria, and conclude with experiments performed to explore how arsenic affects astrocyte metabolism. The results show the astrocyte is dependent upon *de novo* glutathione synthesis and multidrug resistance protein 1 functions during arsenic exposure. Further, the results indicate a shift in astrocyte metabolism toward the production and extrusion of excitotoxic concentrations of glutamate, and demonstrate the astrocyte is dependent upon mitochondrial carbon flux, transamination, and excitatory amino acid transporter 1 activity during arsenic exposure.

1.1 References

Almeida, A., Almeida, J., Bolanos, J.P., and Moncada, S. (2001). Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. *Proc Natl Acad Sci U S A* 98**,** 15294-15299.

Bolanos, J.P., Heales, S.J., Land, J.M., and Clark, J.B. (1995). Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurones and astrocytes in primary culture. *J Neurochem* 64**,** 1965-1972.

Bondy, S.C. (2016). Anthropogenic pollutants may increase the incidence of neurodegenerative disease in an aging population. *Toxicology* 341-343**,** 41-46.

Chakraborti, D., Rahman, M.M., Mukherjee, A., Alauddin, M., Hassan, M., Dutta, R.N., Pati, S., Mukherjee, S.C., Roy, S., Quamruzzman, Q., Rahman, M., Morshed, S., Islam, T., Sorif, S., Selim, M., Islam, M.R., and Hossain, M.M. (2015). Groundwater arsenic contamination in Bangladesh-21 Years of research. *J Trace Elem Med Biol* 31**,** 237-248.

Chattopadhyay, S., Bhaumik, S., Nag Chaudhury, A., and Das Gupta, S. (2002a). Arsenic induced changes in growth development and apoptosis in neonatal and adult brain cells in vivo and in tissue culture. *Toxicol Lett* 128**,** 73-84.

Chattopadhyay, S., Bhaumik, S., Purkayastha, M., Basu, S., Nag Chaudhuri, A., and Das Gupta, S. (2002b). Apoptosis and necrosis in developing brain cells due to arsenic toxicity and protection with antioxidants. *Toxicol Lett* 136**,** 65-76.

Dong, J., and Su, S.Y. (2009). The association between arsenic and children's intelligence: a meta-analysis. *Biol Trace Elem Res* 129**,** 88-93.

Duker, A.A., Carranza, E.J., and Hale, M. (2005). Arsenic geochemistry and health. *Environ Int* 31**,** 631-641.

Flanagan, S.V., Johnston, R.B., and Zheng, Y. (2012). Arsenic in tube well water in Bangladesh: health and economic impacts and implications for arsenic mitigation. *Bull World Health Organ* 90**,** 839-846.

Hamadani, J.D., Tofail, F., Nermell, B., Gardner, R., Shiraji, S., Bottai, M., Arifeen, S.E., Huda, S.N., and Vahter, M. (2011). Critical windows of exposure for arsenic-associated impairment of cognitive function in pre-school girls and boys: a population-based cohort study. *Int J Epidemiol* 40**,** 1593-1604.

Kaler, S., Dhar, P., Bhattacharya, A., and Mehra, R.D. (2013). Preliminary morphological and immunohistochemical changes in rat hippocampus following postnatal exposure to sodium arsenite. *Toxicol Int* 20**,** 160-169.

Namgung, U., and Xia, Z. (2001). Arsenic induces apoptosis in rat cerebellar neurons via activation of JNK3 and p38 MAP kinases. *Toxicol Appl Pharmacol* 174**,** 130-138.

Nordstrom, D.K. (2002). Public health. Worldwide occurrences of arsenic in ground water. *Science* 296**,** 2143-2145.

Prakash, C., Soni, M., and Kumar, V. (2016). Mitochondrial oxidative stress and dysfunction in arsenic neurotoxicity: A review. *J Appl Toxicol* 36**,** 179-188.

Rock, K.D., and Patisaul, H.B. (2018). Environmental Mechanisms of Neurodevelopmental Toxicity. *Curr Environ Health Rep* 5**,** 145-157.

Rosado, J.L., Ronquillo, D., Kordas, K., Rojas, O., Alatorre, J., Lopez, P., Garcia-Vargas, G., Del Carmen Caamano, M., Cebrian, M.E., and Stoltzfus, R.J. (2007). Arsenic exposure and cognitive performance in Mexican schoolchildren. *Environ Health Perspect* 115**,** 1371-1375.

Smith, A.H., Lingas, E.O., and Rahman, M. (2000). Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bull World Health Organ* 78**,** 1093-1103.

Sohel, N., Persson, L.A., Rahman, M., Streatfield, P.K., Yunus, M., Ekstrom, E.C., and Vahter, M. (2009). Arsenic in drinking water and adult mortality: a population-based cohort study in rural Bangladesh. *Epidemiology* 20**,** 824-830.

Supplie, L.M., Duking, T., Campbell, G., Diaz, F., Moraes, C.T., Gotz, M., Hamprecht, B., Boretius, S., Mahad, D., and Nave, K.A. (2017). Respiration-Deficient Astrocytes Survive As Glycolytic Cells In Vivo. *J Neurosci* 37**,** 4231-4242.

Wang, X., Meng, D., Chang, Q., Pan, J., Zhang, Z., Chen, G., Ke, Z., Luo, J., and Shi, X. (2010). Arsenic inhibits neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway. *Environ Health Perspect* 118**,** 627-634.

Wasserman, G.A., Liu, X., Loiacono, N.J., Kline, J., Factor-Litvak, P., Van Geen, A., Mey, J.L., Levy, D., Abramson, R., Schwartz, A., and Graziano, J.H. (2014). A cross-sectional study of well water arsenic and child IQ in Maine schoolchildren. *Environ Health* 13**,** 23.

CHAPTER 2

MITOCHONDRIAL DYSFUNCTION IN GLIAL CELLS: IMPLICATIONS FOR NEURONAL HOMEOSTASIS AND SURVIVAL

(Published November 1 2017 in Toxicology, Vol. 391:109-115)

2.1 Introduction

Mitochondria are involved in a myriad of other processes relevant for cell function besides energy (ATP) production (Yin et al., 2014), making them more than simply powerhouses of the cell. Mitochondria are a hub for signaling processes that include the maintenance of calcium (Ca^{2+}) homeostasis and the formation of signaling molecules and thus, signaling events (Bonini;Chandel, 2015). For example, cell death progression is well known to be triggered by the release of mitochondrial pro-death proteins. Alterations in mitochondrial functions are expected to have important implications for cellular function and disease progression. Correspondingly, numerous pathological conditions have been connected to mitochondrial dysfunction.

Neuronal cell death in brain disorders (neurodegeneration) and injury (neurotoxicity and ischemia) has been linked to a variety of alterations in mitochondrial homeostasis/function including traffic, quality control and turnover, homeostasis (bioenergetics and electron transport) and signaling (metabolism and Ca^{2+} handling) (Chaturvedi and Flint Beal, 2013;Yin et al., 2014). Compared to other cell types, neurons are more dependent on mitochondrial oxidative phosphorylation (OXPHOS) to fulfill their energy demands. Mitochondrial dysfunction with the concomitant energy failure and increased generation of reactive oxygen species (ROS) are considered central to neuronal cell loss in brain disorders because neurons have a limited capacity to upregulate glycolysis or to counteract oxidative damage (Herrero-Mendez et al., 2009;Fernandez-Fernandez et al., 2012). As such, research has been primarily directed at

understanding the causes and consequences of mitochondrial dysfunction in neuronal populations affected during neurodegeneration or brain injury (Moran et al., 2012;Yin et al., 2014).

While initially considered as accessory cells to neurons, glial cells are now recognized to be essential for neuronal cell homeostasis, survival and proper brain function and development (Fernandez-Fernandez et al., 2012;Kubik and Philbert, 2015;Bolanos, 2016). Importantly, genetic modifications or xenobiotics (i.e. pesticides [rotenone or paraquat], metals [lead, arsenic], antibiotics and drugs that target the integrity of mitochondrial DNA) recognized to alter mitochondrial function in neurons are expected to alter mitochondrial function in glial cells as well (Ballinger;Chan;Meyer et al., 2013;Kubik and Philbert, 2015). Unfortunately, very few studies have addressed the pathological implications of mitochondrial dysfunction in glial cells and its consequences in neurological disorders. Herein, we review the current evidence demonstrating the importance of mitochondrial homeostasis and signaling in glial function and how their functional deficiency has important implications for brain disorders and injury that lead to or are a consequence of neuronal cell death.

2.2 Glial cell types and their functional roles

Glial cells can be generally classified as macroglia (astrocytes and oligodendrocytes) or microglia. Macroglia originate from the embryonic ectoderm, while microglia originate from the mesoderm and enter the vertebrate brain during embryogenesis. While initially grouped under the term “glia” (Greek term for glue), it is now clearly established that glial cells regulate a number of physiological processes required for proper neuronal survival and brain function. Refinement and revision of counting techniques have demonstrated that while the overall ratio of neurons to glial varies between different regions in the brain, a ratio of ~1:1 glia to neuron exists in the

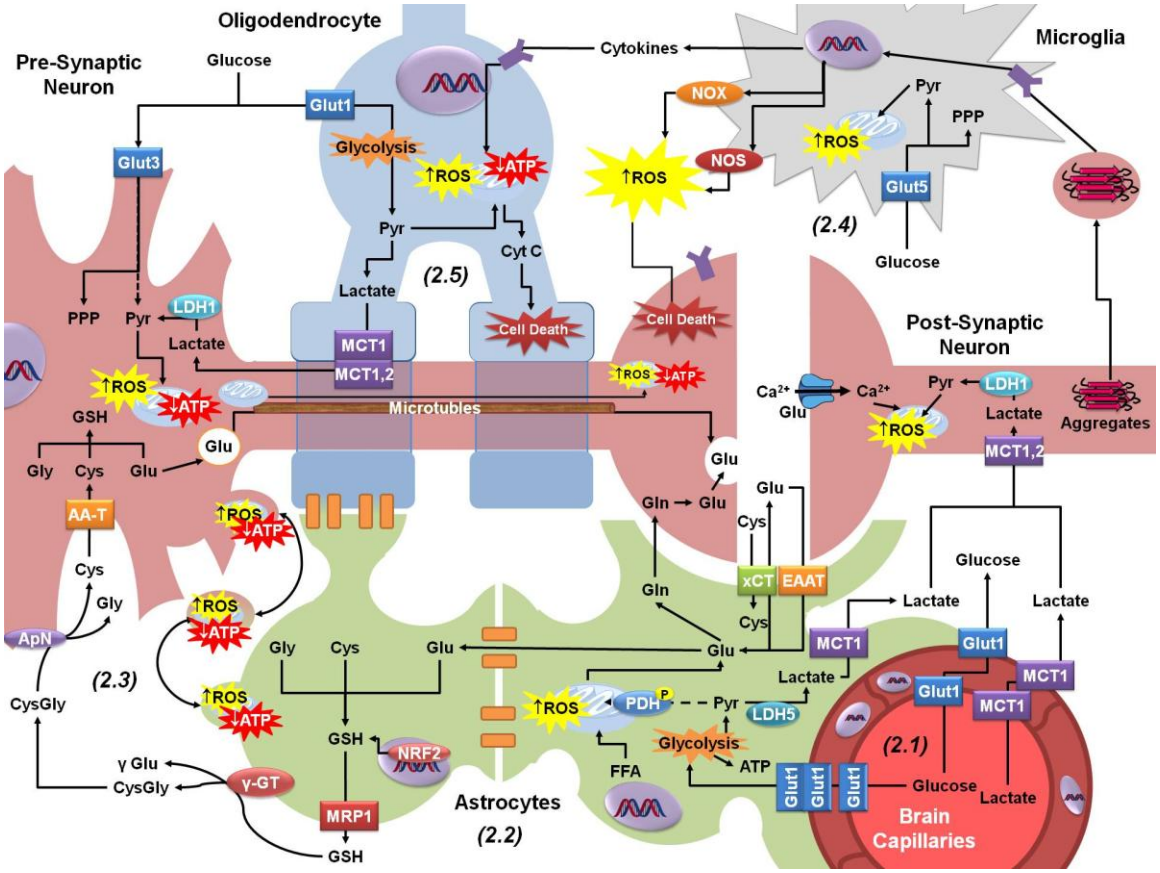


Figure 2.1 Neuronal metabolism, redox homeostasis and signaling are supported by neighboring glial cells.

(1.1) Glucose and lactate enter the brain through Glut1 (glucose transporter 1) and MCT1 (monocarboxylate transporter 1) transporters in the vascular epithelium. Glucose (Glut3) and lactate (MCT1 or 2) are uptaken from the extracellular space by neuronal calls to fuel the TCA cycle for the generation of ATP and biosynthesis of essential molecules.

(1.2) As a component of the blood brain barrier (BBB), astrocytes uptake glucose from the capillary epithelium via Glut1 as well, converting the majority of pyruvate (Pyr) generated into lactate which is exported by MCT1. Astrocytes also uptake the neurotransmitter glutamate (Glu) from the synaptic cleft via EAAT (excitatory amino acid transporters) to be (a) converted into glutamine (Gln), (b) exchanged for extracellular cystine (Cys) by xCT, (c) feed into the TCA cycle, or (d) for GSH synthesis. Astrocytes form

(Figure 2.1 Neuronal metabolism, redox homeostasis and signaling are supported by neighboring glial cells continued)

extended networks with other glia (oligodendrocytes and astrocytes) via gap junctions, sharing nutrients and molecular components with cells more distal to the capillaries.

(1.3) Astrocytes contribute to the redox state of neuronal cells by exporting GSH via MRP1 which is broken down by γGT and ApN into its amino acid components to be uptaken and reassembled as GSH in neuronal cells. Dysfunctional or damaged mitochondrial, likely capable of generating ROS, are transferred from neurons to astrocytes to be degraded by mitophagy.

(1.4) Oligodendrocytes wrap neuronal projections (myelin sheaths) improving signal conduction and like astrocytes, have been proposed to shuttle lactate to the neurons.

(1.5) Microglia are activated by a variety of factors, including cytokines, oxidized proteins, and protein aggregates. Activated microglia migrate to the site of damage and can induce neuronal or oligodendrocyte cell death through the release of cytokines, and the generation of ROS via NADPH oxidases (NOX) and nitric oxide synthases (NOS). AA-T, amino acid transporters; LDH1 or 5, lactate dehydrogenase isoform 1 or 5.

entire human brain, which is significantly smaller than previous estimates (~10:1). Oligodendrocytes are reported to be the most abundant type of glial cells (45–75%), followed by astrocytes (19–40%), and microglia (10% or less) (von Bartheld et al., 2016).

Oligodendrocytes are responsible for axon myelination at large membrane extensions, providing axons with an "insulating coat" that enhances nerve impulse conduction (**Figure 2.1.4**). Oligodendrocytes have several extensions that form several internodal segments of myelin separated by gaps (Ranvier nodes) (Baumann and Pham-Dinh, 2001;Snell, 2010). Oligodendrocytes are found in both gray and white matter, but are a major fraction of all the cells in white matter.

Astrocytes are small cells with processes that are radially arranged, and have considerable molecular, structural, and functional diversity at the regional level. Astrocyte extensions cover the external surface of brain capillaries (perivascular feet), the synaptic cleft between the pre-synaptic and the post-synaptic terminals, and the bare segments of axons at the Ranvier nodes (**Figure 2.1.2**). Astrocytes also form highly organized domains interconnected via gap junctions with other astrocytes and oligodendrocytes (**Figure 2.1.2**). Additionally, astrocytes regulate neurotransmitter levels in the synaptic cleft, provide neurons with energetic and antioxidant precursors (**Figure 2.1.2**), play an important role in neuro/synaptogenesis and tissue repair, and also regulate blood flow and inflammatory processes by the release of signaling mediators (Sofroniew and Vinters, 2010).

Microglial cells are resident macrophages distributed throughout the central nervous system (CNS) (Byrne and Roberts, 2009). As innate immune cells, microglia are activated by infection, tissue injury, or xenobiotics. Upon activation, microglia cells retract their cytoplasmic extensions and migrate to the site of injury, where they proliferate and become antigen presenting

cells. Microglia phagocytose degenerating cells and act as sources of immunoregulatory and neuromodulatory factors such as cytokines, chemokines and neurotrophic factors. Microglia can be activated by cell-surface receptors for endotoxins, cytokines, chemokines, misfolded proteins, serum factors and ATP (**Figure 2.1.5**). While mild activation is a key adaptive immune response, continuous activation or overactivation of microglia is thought to contribute to neurodegeneration (Hanisch and Kettenmann, 2007;Finsen and Owens, 2011;Hanisch, 2013).

2.3 Mitochondrial dysfunction in glial cells and its effect on neuronal function/survival

2.3.1 Cell death

Apoptosis is a ubiquitous homeostatic mechanism critical for the turnover of cells throughout the lifespan of multi-cellular organisms. However, dysregulation of apoptosis occurs as either a cause or consequence of distinct pathologies that include neurodegenerative disorders (Fadeel and Orrenius, 2005). The signaling pathways that regulate the progression of apoptosis have been extensively characterized and divided in two pathways. Induction of apoptosis via the extrinsic pathway is triggered by the activation of the death receptors leading to the activation of initiator caspases. (Lavrik et al., 2005).

The intrinsic mitochondrial pathway of apoptosis is activated by a wide variety of stimuli that regulate the expression and function of the Bcl-2 (B-cell lymphoma 2) family of (anti or pro) apoptotic proteins. The BH3-only Bcl-2 family members (Bad, Bid, Bim and NOXA) regulate the anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xl and Mcl-1) to promote apoptosis. The pro-apoptotic effector proteins Bax and Bak are sufficient and necessary for inducing the permeabilization of the outer mitochondrial membrane and the release of Cyt C (**Figure 2.2.6**). However, the activation of BH3-only proteins derepresses the direct inhibition of Bax and Bak by anti-

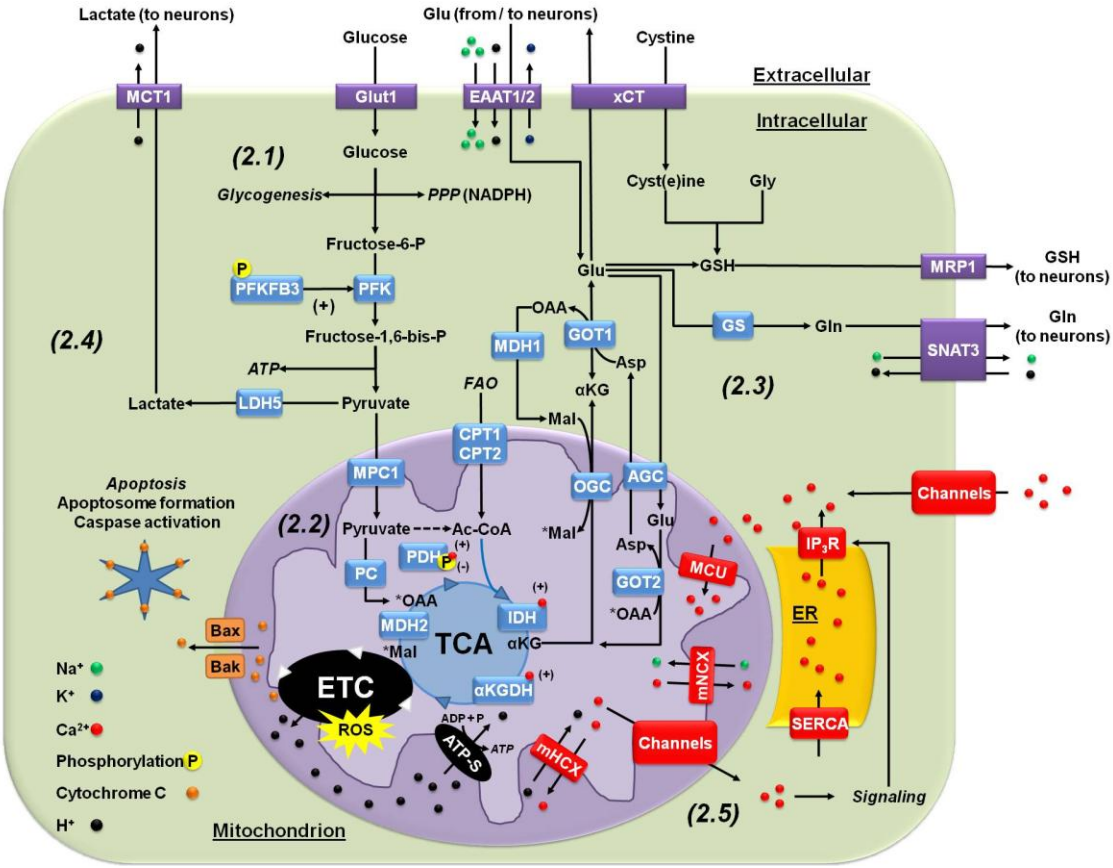


Figure 2.2 Mitochondrial metabolism and signaling in astrocytes.

(2.1) Glucose in astrocytes is used for glycogenesis, NADPH production through the PPP, or glycolysis. Astrocytes are highly glycolytic due to the expression of high levels of 6-phosphofructo-2-kinase / fructose-2,6-bisphosphatase-3 (PFKFB3), whose byproduct fructose-2,6-bisphosphate (F2,6P2), is a positive effector of the glycolytic enzyme 6-phosphofructo-1-kinase (PFK1). In addition, the activity of PFKFB3 is increased by phosphorylation by 5′-AMP-activated protein kinase (AMPK) (Bolanos, 2016).

(2.2) Astrocytes primarily derive ATP from glycolysis rather than oxidative phosphorylation, where pyruvate is converted to lactate by LDH5 and exported to the extracellular space to be consumed by neurons.

(Figure 2.2 Mitochondrial metabolism and signaling in astrocytes continued)

(2.3) Astrocytes carboxylate pyruvate to oxaloacetate (OAA) via pyruvate carboxylase (PC) to regenerate TCA cycle intermediates. Phosphorylation of pyruvate dehydrogenase (PDH) restricts the conversion of pyruvate to acetyl-CoA (Ac-CoA). Thus, FAO has been proposed to be the primary contributor of Ac-CoA to the TCA cycle.

(2.4) α-ketoglutarate (αKG) generated from the TCA cycle can be transported to the cytosol and converted to Glu by glutamic-oxaloacetic transaminase 1 or aspartate (Asp) aminotransferase (GOT1) as part of the malate-Asp shuttle. Glu has three central metabolic pathways in astrocytes. 1) Glu can be converted to Gln by GS and exported to neurons by the sodium-coupled neutral amino acid transporter 3 (SNAT3). 2) Glu is exchanged via xCT for extracellular cystine that is reduced to Cys. Extracellular Glu can be uptaken back by astrocytes by EAAT1/2. Finally, 3) Glu, Gly and Cys are precursors of GSH, which is also exported to neurons via MRP1.

(2.5) The ER acts as a store for intracellular calcium, where the sarco/endoplasmic reticulum calcium ion ATPase (SERCA) pumps cytosolic Ca^{2+} into the ER. Ca^{2+} signaling is tightly regulated by the activation of IP3R that release Ca^{2+} from ER stores, as well as by the activation of plasma membrane Ca^{2+} channels. Mitochondria can buffer Ca^{2+} by its transport across the inner mitochondrial membrane to the matrix via MCU), while the export is performed by mNCX and mHCX. Mitochondria can also transport Ca^{2+} in and out of the mitochondria via the activation of distinct Ca^{2+} permeable channels. In the matrix, Ca^{2+} stimulates TCA carbon flux by binding to PDH, IDH, and αKGDH, increasing the activity of the ETC and ATP production.

(2.6) Cyt C is held close to the inner mitochondrial membrane by cardiolipin (not shown), acting as a component of ETC. Dissociation of Cyt C from cardiolipin, through oxidative or enzymatic means, coupled with permeabilization of the outer mitochondrial membrane by the formation of Bax/Bak oligomeric channels, allows Cyt C to escape into the cytosol. Cytosolic Cyt C associates with apoptotic protease-

(Figure 2.2 Mitochondrial metabolism and signaling in astrocytes continued)

activating factor 1 (APAF1), forming the apoptosome and leading to the activation of caspases to initiate apoptosis. AGC, aspartate-glutamate carrier; CPT1 or 2, carnitine palmitoyltransferase isoform 1 or 2; MDH1 or 2, malate dehydrogenase isoform 1 or 2; MPC1, mitochondrial pyruvate carrier 1; OGC, 2-oxoglutarate (α-ketoglutarate) carrier.

apoptotic Bcl-2 proteins. Released Cyt C leads to the recruitment of Apaf1 and caspase 9 into a platform (apoptosome) that activates caspase 9 and subsequently, executioner caspases 3, 6 and 7. The extrinsic / death receptor pathway can crosstalk to the intrinsic / mitochondrial pathway of apoptosis by an amplification loop induced by caspase dependent cleavage/activation of Bid (Green and Llambi, 2015).

While a number of studies have reported the induction of apoptosis in astrocytes and microglia under different experimental conditions, very little evidence exists about the loss or degeneration of these glial cells with respect to human disorders. Conversely, oligodendrocytes are known to degenerate in demyelinating disorders such as multiple sclerosis, and to be affected directly or indirectly by the majority of known disorders in the CNS including ischemia, trauma and neurodegeneration. Glutamate/Ca^{2+} excitotoxicity, inflammation (cytokines) and oxidative stress are common triggers for oligodendrocyte injury in these pathological situations (**Figure 2.1.4**). Oligodendrocytes express ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainite receptors whose activation induces Ca^{2+} overflow and apoptotic cell death via the intrinsic mitochondrial pathway via activation of Bax and caspase 3 (**Figure 2.1.4**) (Ruiz et al., 2010;Sanchez-Gomez et al., 2011). The high lipid and iron content of oligodendrocytes also makes them susceptible to oxidative damage induced by cytokines (Zhang et al., 2005).

2.3.2 Bioenergetics and metabolism

Neurons are dependent on high rates of OXPHOS to meet their energy requirements, to maintain and restore ionic gradients, and for the uptake and recycling of neurotransmitters. In contrast, astrocytes are highly glycolytic (**Figure 2.2.1**), but a large portion of glucose is converted to lactate and released to the extracellular space. Interestingly, glucose consumption in astrocytes exceeds their energy expenditure, which is explained by the astrocytes-neuron lactate

shuttle hypothesis where lactate is shuttled from astrocytes (and oligodendrocytes) as a fuel for OXPHOS in neurons (**Figure 2.1.1** and **2.2.2**) (Belanger et al., 2011;Funfschilling et al., 2012a;Lee et al., 2012;Morrison et al., 2013). What limits OXPHOS in astrocytes? Recent studies have demonstrated that the activity of pyruvate dehydrogenase (PDH), which provides a route of entry for pyruvate into the tricarboxylic acid (TCA or Krebs) cycle, is reduced by its phosphorylation in astrocytes (**Figure 2.1.1** and **2.2.3**) (Halim et al., 2010). Interestingly, astrocytes have the same oxidative capacity as neurons, but are resilient to mitochondrial dysfunction (Di Monte et al., 1992).

Other carbon sources can fuel OXPHOS in astrocytes. Glutamate can be metabolized through the TCA cycle, but astrocytes primarily metabolize it to glutamine by the activity of glutamine synthase (GS) (**Figure 2.2.4**). However, when the extracellular concentration of glutamate increases to levels observed during synaptic transmission, the proportion of glutamate metabolized by the TCA cycle increases as well, while its conversion to glutamine decreases concomitantly (McKenna, 2013;Schousboe et al., 2014;Nissen et al., 2015). Importantly, glutamate also exerts a stimulatory effect on glycolysis as well (Pellerin and Magistretti, 1994;Loaiza et al., 2003).

Acetate is also used as a carbon source by astrocytes, but its physiological significance has not been established (Belanger et al., 2011;Jiang et al., 2013). Astrocytes can oxidize free fatty acids (FFA) and ketone bodies, but neurons and oligodendrocytes can only use ketone bodies as these cell types would be highly vulnerable to ROS formation generated by FFA oxidation due to their high lipid content (Schonfeld and Reiser, 2013;Iglesias et al., 2016). Twenty percent of total energy expenditure in the brain is linked to FFA oxidation (FAO), which occurs primarily in astrocytes (Ebert et al., 2003). As mentioned above, astrocytes exhibit high

rates of OXPHOS (Lovatt et al., 2007), but a larger proportion of astrocyte PDH is phosphorylated compared to neuronal PDH, inhibiting the conversion of pyruvate to acetyl-CoA (Halim et al., 2010). Thus, FAO might actually be a major source for acetyl-CoA into the TCA cycle (Panov et al., 2014) (**Figure 2.2.3**).

Oligodendrocytes have similar rates of glycolysis compared to astrocytes, but release less lactate since a larger proportion of pyruvate derived from glucose is metabolized via PDH into the TCA cycle. Similar to astrocytes, oligodendrocytes can carboxylate pyruvate to oxaloacetate via pyruvate carboxylase (PC) to replenish TCA intermediates (anaplerosis) or recycle pyruvate (**Figure 2.2.3**) (Amaral et al., 2016). In astrocytes however, pyruvate carboxylation also serves to compensate for the loss of TCA intermediates due to the generation of glutamate and subsequently glutamine that is then shuttled to neurons (glutamate-glutamine cycle) (**Figure 2.1.2** and **2.2.4**) (Schousboe et al., 2014). Lactate metabolism in oligodendrocytes has been demonstrated to participate in oligodendrocyte differentiation and myelination.(Rinholm et al., 2011). Importantly, mitochondrial respiration / metabolism seems to be primarily involved in oligodendrocyte differentiation, while glycolysis appears to be sufficient to maintain post-myelinated (differentiated) oligodendrocytes (Funfschilling et al., 2012b). Accordingly, demyelination disorders linked to mitochondrial dysfunction seem to be primarily linked to increased oxidative damage and changes in FFA metabolism but not energy failure (Swalwell et al., 2011;Lin et al., 2012;Viader et al., 2013).

2.3.3 Calcium

Calcium (Ca^{2+}) signaling is tightly coupled to its homeostasis. Ca^{2+} gradients across membranes and cellular compartments are established by the activity of Ca^{2+} pumps / transporters. The controlled activation of Ca^{2+} fluxes allows its release and the subsequent

activation of a diverse array of signal transducers including kinases, enzymes and ion channels. Mitochondria are now recognized as important Ca^{2+} reservoirs or sinks. The regulation of Ca^{2+} signaling is not a simple process of its release and subsequent compartmentalization. Instead, it involves a highly localized release and controlled diffusion of Ca^{2+} across intracellular compartments and in most cases, the coordinated action of more than one Ca^{2+} reservoir and release / uptake system. The spatiotemporal complexity of this process is reflected by the existence of patterns of Ca^{2+} waves or sparks that are decoded by transducers selectively localized in different cellular compartments. Sequestration of Ca^{2+} within the mitochondrial matrix is partially driven by the negative environment generated by the extrusion of protons (H^+) across the inner mitochondrial membrane by the ETC (**Figure 2.2.3**). Translocation of Ca^{2+} into the matrix is mediated by the mitochondrial Ca^{2+} uniporter (MCU) in an energy-independent manner (**Figure 2.2.5**). Ca^{2+} release from the mitochondria is mediated by Ca^{2+} exchangers (the sodium $[Na^+)]/Ca^{2+}$ [mNCX] and mitochondrial proton $[H^+]/Ca^{2+}$ exchangers [mHCX]), or the opening of the mitochondrial permeability transition pore under pathological conditions (**Figure 2.2.5**). Importantly, mitochondria act as important buffers for Ca^{2+} release / influx from the endoplasmic reticulum (ER) and the plasma membrane that contribute to the regulation of Ca^{2+} signaling (**Figure 2.2.5**) (Rizzuto et al., 2012).

Very little is known about the impact of mitochondrial Ca^{2+} homeostasis on glial signaling. However, as in other cell types, functional mitochondria in astrocytes and oligodendrocytes regulates Ca^{2+} waves generated by the activation of inositol 1,4,5-triphosphate (IP3) receptors (IP3R) and the release of Ca^{2+} from the ER (Simpson and Russell, 1996;Boitier et al., 1999;Smith et al., 2005). Mitochondrial Ca^{2+} has also been shown to regulate vesicular glutamate release from astrocytes that modulates synaptic communication and excitability (Reyes and Parpura, 2008). Ca^{2+} accumulation in mitochondria also modulates oxidative phosphorylation

and energy production. PDH activity is regulated by a Ca^{2+}-dependent dephosphorylation, while Ca^{2+} binding also regulates α-ketoglutarate (αKGDH)- and isocitrate (IDH)-dehydrogenase activity, which increases NADH levels, electron flow and ATP synthesis (**Figure 2.2.5**) (Rizzuto et al., 2012). Accordingly, Ca^{2+} release from the ER stimulates mitochondrial-dependent energy production in astrocytes (Wu et al., 2007). Not only do mitochondria regulate Ca^{2+} accumulation and dynamics, but also its release. A recent report demonstrated that Ca^{2+} release via mNCX is coupled to store-operated Ca^{2+} entry (triggered by Ca^{2+} depletion from ER stores) and regulates astrocytes proliferation and excitotoxic glutamate release (Parnis et al., 2013). In microglia, mitochondrial Ca^{2+} influx via the mitochondrial transient receptor potential vanilloid 1 channel (TRPV1) depolarizes mitochondria resulting in mtROS production, mitogen activated protein kinase (MAPK) activation, and enhanced migration (Miyake et al., 2015).

2.3.4 Inflammation

Inflammation is a key contributor to most neurological disorders. In a steady "basal" state, microglia performs continuous surveillance of the CNS, secrete neurotrophic factors, such as insulin-like growth factor 1 (IGF1), brain-derived neurotrophic factor (BDNF), transforming growth factor-β (TGFβ) and nerve growth factor (NGF), and promote synapse pruning for refinement of neuronal circuits during development. Classical activation of microglia (M1) conveys the production of ROS and nitrogen species (RNS) and the release of pro-inflammatory cytokines (tumor necrosis factor [TNF] and interleukin-1β [IL-1β]) to promote brain tissue repair upon injury (removal of cell debris and restoring of tissue integrity) and, upon prolonged activation, neuronal dysfunction as well. Disease-associated factors such as xenobiotics, protein aggregates, and damage (DAMPs) or pathogen-associated molecular patterns (PAMPS) can activate microglia through a variety of surface receptors. These receptors include Toll-like

receptors (for lipopolysacharide [LPS], oxidized low-density lipoprotein [LDL] and molecules released by damaged or dead cells including high-mobility group box 1 [HMGB1] and nucleotides), nucleotide-binding oligomerization domain (Nod)-like receptors (for amyloid proteins), advanced glycation end-products receptors or RAGE (that are also activated by HMGB1), and purinergic receptors (for purines and pyrimidines including nucleoside triphosphates, e.g. ATP) (Hu et al., 2014). Pro-inflammatory cytokines released from microglia also "activate" astrocytes, which might produce TNF to potentiate microglia activation as well. As such, co-cultures of microglia and astrocytes produce more neurotoxic factors than either activated cell type alone (Saijo and Glass, 2011). Whether astrocytes can be activated in the absence of microglia is still unclear since most studies using primary cultures of astrocytes also contain at least 5% of microglia that significantly contribute to astrocyte activation (Facci et al., 2014;Marinelli et al., 2015). The alternative (M2-like) phenotype of microglia is observed to be induced by transforming growth factor-β (TGFβ), IL-4, IL-6 and IL-10 secreted from glioma cells (Saijo and Glass, 2011).

Mitochondrial dysfunction triggers inflammatory responses (West). During inflammation, changes in mitochondrial metabolism contribute to the activation of microglia. The M1 phenotype of microglia was recently reported to be paralleled by a metabolic switch from mitochondrial OXPHOS to glycolysis that enhances carbon flux to the PPP (**Figure 2.1.5**) (Voloboueva et al., 2013;Gimeno-Bayon et al., 2014;Orihuela et al., 2016). Interestingly, inhibition of complex I activity activates microglial cells (Shaikh and Nicholson, 2009;Yuan et al., 2013;Ye et al., 2016), while impairment of mitochondrial fission reduces the production of pro-inflammatory signals (Park et al., 2013). Induction of the M2-like phenotype results in no observable changes in mitochondrial oxygen consumption or lactate production (Orihuela et al., 2016). However, mitochondrial toxins such as 3-nitropropionic acid and rotenone impair the

transition to the M2 phenotype induced by IL-4 (Ferger et al., 2010). These results suggest that mitochondrial dysfunction in microglia can exacerbate the pro-inflammatory M1 phenotype and result in the release of neurotoxic pro-inflammatory cytokines, and enhanced ROS / RNS formation (Tang and Le, 2016).

2.3.5 Redox homeostasis and detoxification of xenobiotics

In general, neurons have limited defense mechanisms against ROS compared to astrocytes. This enhanced resistance to oxidative damage in astrocytes is observed despite the fact that astrocytes have a deficient mitochondrial respiration and increased ROS formation when compared to neurons (Lopez-Fabuel et al., 2016). A comparative study also demonstrated that astrocytes are more resistant to oxidative damage than microglia or oligodendrocytes (Hollensworth et al., 2000). Astrocytes contain higher levels of endogenous antioxidants and antioxidant systems that include NADPH and G6PD (glucose-6-phosphate dehydrogenase). Astrocytes' resistance to oxidative damage is explained by the activation of the antioxidant response via the nuclear factor erythroid-2-related factor 2 (Nrf2) transcription factor (Garcia-Nogales et al., 2003;Shih et al., 2003). Both neurons and astrocytes can synthesize GSH, but neurons depend on the supply of GSH precursors from astrocytes (**Figure 2.1.3**). GSH is released from astrocytes via the ATP-binding cassette transporters subfamily C member 1 transporter (ABCC1, or multidrug-resistance-associated protein 1 [MRP1]) (Hirrlinger and Dringen, 2005). Extracellular GSH is then degraded by the γ-glutamyl transpeptidase (γGT) to produce l-cysteine-l-glycine (CysGly), which is cleaved further by the neuronal aminopeptidase N (ApN) into the amino acids glycine and cysteine that are taken up by neurons for *de novo* GSH synthesis (**Figure 2.1.3**) (Aoyama et al., 2008;Belanger et al., 2011). The glutamate-glutamine cycle might also be involved in the regulation of the neuronal redox environment by astrocytes since GSH synthesis

also requires glutamate. The importance of astrocytes for neuronal redox homeostasis was evidenced by a recent study demonstrating that conditional depletion of astrocytes promotes neuronal injury by oxidative stress (Schreiner et al., 2015). Astrocytes are also the first line of defense against xenobiotics entering into the brain since their extensions cover the external surface of capillaries as part of the blood brain barrier. Detoxification of electrophiles is dependent the formation of irreversible adducts with GSH that in many cases depends on the activity of glutathione-S-transferases (GST) and their efflux through MRPs (Dringen et al., 2015).

But what is the role of mitochondria in redox homeostasis in astrocytes and neurons? The loss of GSH by its export to neurons or due to the detoxification of electrophiles is expected to prompt astrocytes to replenish GSH precursors. Interestingly, GSH depletion upregulates mitochondrial activity in astrocytes (Vasquez et al., 2001) and we have recently observed that mitochondrial OXPHOS is essential for the detoxification of electrophiles via the GSH/MRP system (*manuscript in preparation*), but the exact mechanisms that regulate this phenomenon are still unclear.

2.4 Conclusions and Perspectives

Mitochondrial dysfunction has been widely recognized as central to the pathogenesis of neurological disorders. However, the majority of current research efforts have been focused on understanding the causes and consequences of mitochondrial dysfunction in neuronal cells that rely on OXPHOS to generate energy and are also more sensitive to mitochondrial ROS formation. Less is known about the functional role of mitochondria in glial cells and its implications for neuronal survival and brain function. In this work, we have provided an overview of the role of mitochondria in glial cell function that includes metabolism, redox homeostasis, Ca^{2+} signaling, inflammation and cell death. The evidence so far clearly demonstrates the

importance of mitochondrial health in glial cells and its relevance to neuronal function. Nevertheless, this review also highlights our limited understanding of mitochondria function in glial cells and the need for further investigations in this area that is expanding. For example, recent studies have demonstrated that damaged mitochondria can be transferred from neuronal axons for their turnover in astrocytes (Davis et al., 2014), and conversely, astrocytes have been shown to transfer mitochondria to promote neuronal survival (Hayakawa et al., 2016) (**Figure 2.1.3**). Many questions remain to be answered regarding the role of mitochondrial in neurological disorders, but it is time for us to think about mitochondrial health and dysfunction in a more inclusive context outside neuronal cells.

2.5 Acknowledgements

This work was supported by the National Institutes of Health Grants P20RR17675 Centers of Biomedical Research Excellence (COBRE), and the Office of Research of the University of Nebraska-Lincoln.

2.5 References

Amaral, A.I., Hadera, M.G., Tavares, J.M., Kotter, M.R., and Sonnewald, U. (2016). Characterization of glucose-related metabolic pathways in differentiated rat oligodendrocyte lineage cells. *Glia* 64**,** 21-34.

Aoyama, K., Watabe, M., and Nakaki, T. (2008). Regulation of neuronal glutathione synthesis. *J Pharmacol Sci* 108**,** 227-238.

Ballinger, S. Mitochondrial Toxicity of Tobacco Smoke and Air Pollution. *This Special Issue*.

Baumann, N., and Pham-Dinh, D. (2001). Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev* 81**,** 871-927.

Belanger, M., Allaman, I., and Magistretti, P.J. (2011). Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. *Cell Metab* 14**,** 724-738.

Boitier, E., Rea, R., and Duchen, M.R. (1999). Mitochondria exert a negative feedback on the propagation of intracellular Ca2+ waves in rat cortical astrocytes. *J Cell Biol* 145**,** 795-808.

Bolanos, J.P. (2016). Bioenergetics and redox adaptations of astrocytes to neuronal activity. *J Neurochem* 139 Suppl 2**,** 115-125.

Bonini, M. Oxidative signaling in mitochondrial homeostasis. *This Special Issue*.

Byrne, J.H., and Roberts, J.L. (2009). *From molecules to networks : an introduction to cellular and molecular neuroscience.* Amsterdam ; Boston: Academic Press/Elsevier.

Chan, S.S. Inherited mitochondrial genomic instability and chemical exposures *This Special Issue*.

Chandel, N.S. (2015). Evolution of Mitochondria as Signaling Organelles. *Cell Metab* 22**,** 204-206.

Chaturvedi, R.K., and Flint Beal, M. (2013). Mitochondrial diseases of the brain. *Free Radic Biol Med* 63**,** 1-29.

Davis, C.H., Kim, K.Y., Bushong, E.A., Mills, E.A., Boassa, D., Shih, T., Kinebuchi, M., Phan, S., Zhou, Y., Bihlmeyer, N.A., Nguyen, J.V., Jin, Y., Ellisman, M.H., and Marsh-Armstrong, N. (2014). Transcellular degradation of axonal mitochondria. *Proc Natl Acad Sci U S A* 111**,** 9633-9638.

Di Monte, D.A., Wu, E.Y., Delanney, L.E., Irwin, I., and Langston, J.W. (1992). Toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in primary cultures of mouse astrocytes. *J Pharmacol Exp Ther* 261**,** 44-49.

Dringen, R., Brandmann, M., Hohnholt, M.C., and Blumrich, E.M. (2015). Glutathione-Dependent Detoxification Processes in Astrocytes. *Neurochem Res* 40**,** 2570-2582.

Ebert, D., Haller, R.G., and Walton, M.E. (2003). Energy contribution of octanoate to intact rat brain metabolism measured by 13C nuclear magnetic resonance spectroscopy. *J Neurosci* 23**,** 5928-5935.

Facci, L., Barbierato, M., Marinelli, C., Argentini, C., Skaper, S.D., and Giusti, P. (2014). Toll-like receptors 2, -3 and -4 prime microglia but not astrocytes across central nervous system regions for ATP-dependent interleukin-1beta release. *Sci Rep* 4**,** 6824.

Fadeel, B., and Orrenius, S. (2005). Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med* 258**,** 479-517.

Ferger, A.I., Campanelli, L., Reimer, V., Muth, K.N., Merdian, I., Ludolph, A.C., and Witting, A. (2010). Effects of mitochondrial dysfunction on the immunological properties of microglia. *J Neuroinflammation* 7**,** 45.

Fernandez-Fernandez, S., Almeida, A., and Bolanos, J.P. (2012). Antioxidant and bioenergetic coupling between neurons and astrocytes. *Biochem J* 443**,** 3-11.

Finsen, B., and Owens, T. (2011). Innate immune responses in central nervous system inflammation. *FEBS Lett* 585**,** 3806-3812.

Funfschilling, U., Supplie, L.M., Mahad, D., Boretius, S., Saab, A.S., Edgar, J., Brinkmann, B.G., Kassmann, C.M., Tzvetanova, I.D., Mobius, W., Diaz, F., Meijer, D., Suter, U., Hamprecht, B., Sereda, M.W., Moraes, C.T., Frahm, J., Goebbels, S., and Nave, K.-A. (2012a). Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature* 485**,** 517-521.

Funfschilling, U., Supplie, L.M., Mahad, D., Boretius, S., Saab, A.S., Edgar, J., Brinkmann, B.G., Kassmann, C.M., Tzvetanova, I.D., Mobius, W., Diaz, F., Meijer, D., Suter, U., Hamprecht, B., Sereda, M.W., Moraes, C.T., Frahm, J., Goebbels, S., and Nave, K.A. (2012b). Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature* 485**,** 517-521.

Garcia-Nogales, P., Almeida, A., and Bolanos, J.P. (2003). Peroxynitrite protects neurons against nitric oxide-mediated apoptosis. A key role for glucose-6-phosphate dehydrogenase activity in neuroprotection. *J Biol Chem* 278**,** 864-874.

Gimeno-Bayon, J., Lopez-Lopez, A., Rodriguez, M.J., and Mahy, N. (2014). Glucose pathways adaptation supports acquisition of activated microglia phenotype. *J Neurosci Res* 92**,** 723-731.

Green, D.R., and Llambi, F. (2015). Cell Death Signaling. *Cold Spring Harb Perspect Biol* 7.

Halim, N.D., Mcfate, T., Mohyeldin, A., Okagaki, P., Korotchkina, L.G., Patel, M.S., Jeoung, N.H., Harris, R.A., Schell, M.J., and Verma, A. (2010). Phosphorylation status of pyruvate dehydrogenase distinguishes metabolic phenotypes of cultured rat brain astrocytes and neurons. *Glia* 58**,** 1168-1176.

Hanisch, U.K. (2013). Proteins in microglial activation--inputs and outputs by subsets. *Curr Protein Pept Sci* 14**,** 3-15.

Hanisch, U.K., and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 10**,** 1387-1394.

Hayakawa, K., Esposito, E., Wang, X., Terasaki, Y., Liu, Y., Xing, C., Ji, X., and Lo, E.H. (2016). Transfer of mitochondria from astrocytes to neurons after stroke. *Nature* 535**,** 551-555.

Herrero-Mendez, A., Almeida, A., Fernandez, E., Maestre, C., Moncada, S., and Bolanos, J.P. (2009). The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nat Cell Biol* 11**,** 747-752.

Hirrlinger, J., and Dringen, R. (2005). Multidrug resistance protein 1-mediated export of glutathione and glutathione disulfide from brain astrocytes. *Methods Enzymol* 400**,** 395-409.

Hollensworth, S.B., Shen, C., Sim, J.E., Spitz, D.R., Wilson, G.L., and Ledoux, S.P. (2000). Glial cell type-specific responses to menadione-induced oxidative stress. *Free Radic Biol Med* 28**,** 1161-1174.

Hu, X., Liou, A.K., Leak, R.K., Xu, M., An, C., Suenaga, J., Shi, Y., Gao, Y., Zheng, P., and Chen, J. (2014). Neurobiology of microglial action in CNS injuries: receptor-mediated signaling mechanisms and functional roles. *Prog Neurobiol* 119-120**,** 60-84.

Iglesias, J., Morales, L., and Barreto, G.E. (2016). Metabolic and Inflammatory Adaptation of Reactive Astrocytes: Role of PPARs. *Mol Neurobiol*.

Jiang, L., Gulanski, B.I., De Feyter, H.M., Weinzimer, S.A., Pittman, B., Guidone, E., Koretski, J., Harman, S., Petrakis, I.L., Krystal, J.H., and Mason, G.F. (2013). Increased brain uptake and oxidation of acetate in heavy drinkers. *J Clin Invest* 123**,** 1605-1614.

Kubik, L.L., and Philbert, M.A. (2015). The role of astrocyte mitochondria in differential regional susceptibility to environmental neurotoxicants: tools for understanding neurodegeneration. *Toxicol Sci* 144**,** 7-16.

Lavrik, I., Golks, A., and Krammer, P.H. (2005). Death receptor signaling. *J Cell Sci* 118**,** 265-267.

Lee, Y., Morrison, B.M., Li, Y., Lengacher, S., Farah, M.H., Hoffman, P.N., Liu, Y., Tsingalia, A., Jin, L., Zhang, P.W., Pellerin, L., Magistretti, P.J., and Rothstein, J.D. (2012). Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* 487**,** 443-448.

Lin, C.S., Sharpley, M.S., Fan, W., Waymire, K.G., Sadun, A.A., Carelli, V., Ross-Cisneros, F.N., Baciu, P., Sung, E., Mcmanus, M.J., Pan, B.X., Gil, D.W., Macgregor, G.R., and Wallace, D.C. (2012). Mouse mtDNA mutant model of Leber hereditary optic neuropathy. *Proc Natl Acad Sci U S A* 109**,** 20065-20070.

Loaiza, A., Porras, O.H., and Barros, L.F. (2003). Glutamate triggers rapid glucose transport stimulation in astrocytes as evidenced by real-time confocal microscopy. *J Neurosci* 23**,** 7337-7342.

Lopez-Fabuel, I., Le Douce, J., Logan, A., James, A.M., Bonvento, G., Murphy, M.P., Almeida, A., and Bolanos, J.P. (2016). Complex I assembly into supercomplexes determines differential mitochondrial ROS production in neurons and astrocytes. *Proc Natl Acad Sci U S A* 113**,** 13063-13068.

Lovatt, D., Sonnewald, U., Waagepetersen, H.S., Schousboe, A., He, W., Lin, J.H., Han, X., Takano, T., Wang, S., Sim, F.J., Goldman, S.A., and Nedergaard, M. (2007). The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. *J Neurosci* 27**,** 12255-12266.

Marinelli, C., Di Liddo, R., Facci, L., Bertalot, T., Conconi, M.T., Zusso, M., Skaper, S.D., and Giusti, P. (2015). Ligand engagement of Toll-like receptors regulates their expression in cortical microglia and astrocytes. *J Neuroinflammation* 12**,** 244.

Mckenna, M.C. (2013). Glutamate pays its own way in astrocytes. *Front Endocrinol (Lausanne)* 4**,** 191.

Meyer, J.N., Leung, M.C., Rooney, J.P., Sendoel, A., Hengartner, M.O., Kisby, G.E., and Bess, A.S. (2013). Mitochondria as a target of environmental toxicants. *Toxicol Sci* 134**,** 1-17.

Miyake, T., Shirakawa, H., Nakagawa, T., and Kaneko, S. (2015). Activation of mitochondrial transient receptor potential vanilloid 1 channel contributes to microglial migration. *Glia* 63**,** 1870-1882.

Moran, M., Moreno-Lastres, D., Marin-Buera, L., Arenas, J., Martin, M.A., and Ugalde, C. (2012). Mitochondrial respiratory chain dysfunction: implications in neurodegeneration. *Free Radic Biol Med* 53**,** 595-609.

Morrison, B.M., Lee, Y., and Rothstein, J.D. (2013). Oligodendroglia metabolically support axons and maintain structural integrity. *Trends in cell biology* 23**,** 10.1016/j.tcb.2013.1007.1007.

Nissen, J.D., Pajecka, K., Stridh, M.H., Skytt, D.M., and Waagepetersen, H.S. (2015). Dysfunctional TCA-Cycle Metabolism in Glutamate Dehydrogenase Deficient Astrocytes. *Glia* 63**,** 2313-2326.

Orihuela, R., Mcpherson, C.A., and Harry, G.J. (2016). Microglial M1/M2 polarization and metabolic states. *Br J Pharmacol* 173**,** 649-665.

Panov, A., Orynbayeva, Z., Vavilin, V., and Lyakhovich, V. (2014). Fatty acids in energy metabolism of the central nervous system. *Biomed Res Int* 2014**,** 472459.

Park, J., Choi, H., Min, J.S., Park, S.J., Kim, J.H., Park, H.J., Kim, B., Chae, J.I., Yim, M., and Lee, D.S. (2013). Mitochondrial dynamics modulate the expression of pro-inflammatory mediators in microglial cells. *J Neurochem* 127**,** 221-232.

Parnis, J., Montana, V., Delgado-Martinez, I., Matyash, V., Parpura, V., Kettenmann, H., Sekler, I., and Nolte, C. (2013). Mitochondrial exchanger NCLX plays a major role in the intracellular Ca2+ signaling, gliotransmission, and proliferation of astrocytes. *J Neurosci* 33**,** 7206-7219.

Pellerin, L., and Magistretti, P.J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A* 91**,** 10625-10629.

Reyes, R.C., and Parpura, V. (2008). Mitochondria modulate Ca2+-dependent glutamate release from rat cortical astrocytes. *J Neurosci* 28**,** 9682-9691.

Rinholm, J.E., Hamilton, N.B., Kessaris, N., Richardson, W.D., Bergersen, L.H., and Attwell, D. (2011). Regulation of oligodendrocyte development and myelination by glucose and lactate. *J Neurosci* 31**,** 538-548.

Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. *Nat Rev Mol Cell Biol* 13**,** 566-578.

Ruiz, A., Matute, C., and Alberdi, E. (2010). Intracellular Ca2+ release through ryanodine receptors contributes to AMPA receptor-mediated mitochondrial dysfunction and ER stress in oligodendrocytes. *Cell Death Dis* 1**,** e54.

Saijo, K., and Glass, C.K. (2011). Microglial cell origin and phenotypes in health and disease. *Nat Rev Immunol* 11**,** 775-787.

Sanchez-Gomez, M.V., Alberdi, E., Perez-Navarro, E., Alberch, J., and Matute, C. (2011). Bax and calpain mediate excitotoxic oligodendrocyte death induced by activation of both AMPA and kainate receptors. *J Neurosci* 31**,** 2996-3006.

Schonfeld, P., and Reiser, G. (2013). Why does brain metabolism not favor burning of fatty acids to provide energy? Reflections on disadvantages of the use of free fatty acids as fuel for brain. *J Cereb Blood Flow Metab* 33**,** 1493-1499.

Schousboe, A., Scafidi, S., Bak, L.K., Waagepetersen, H.S., and Mckenna, M.C. (2014). Glutamate metabolism in the brain focusing on astrocytes. *Adv Neurobiol* 11**,** 13-30.

Schreiner, B., Romanelli, E., Liberski, P., Ingold-Heppner, B., Sobottka-Brillout, B., Hartwig, T., Chandrasekar, V., Johannssen, H., Zeilhofer, H.U., Aguzzi, A., Heppner, F., Kerschensteiner, M., and Becher, B. (2015). Astrocyte Depletion Impairs Redox Homeostasis and Triggers Neuronal Loss in the Adult CNS. *Cell Rep* 12**,** 1377-1384.

Shaikh, S.B., and Nicholson, L.F. (2009). Effects of chronic low dose rotenone treatment on human microglial cells. *Mol Neurodegener* 4**,** 55.

Shih, A.Y., Johnson, D.A., Wong, G., Kraft, A.D., Jiang, L., Erb, H., Johnson, J.A., and Murphy, T.H. (2003). Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. *J Neurosci* 23**,** 3394-3406.

Simpson, P.B., and Russell, J.T. (1996). Mitochondria support inositol 1,4,5-trisphosphate-mediated Ca2+ waves in cultured oligodendrocytes. *J Biol Chem* 271**,** 33493-33501.

Smith, I.F., Boyle, J.P., Kang, P., Rome, S., Pearson, H.A., and Peers, C. (2005). Hypoxic regulation of Ca2+ signaling in cultured rat astrocytes. *Glia* 49**,** 153-157.

Snell, R.S. (2010). *Clinical neuroanatomy.* Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Sofroniew, M.V., and Vinters, H.V. (2010). Astrocytes: biology and pathology. *Acta Neuropathol* 119**,** 7-35.

Swalwell, H., Kirby, D.M., Blakely, E.L., Mitchell, A., Salemi, R., Sugiana, C., Compton, A.G., Tucker, E.J., Ke, B.X., Lamont, P.J., Turnbull, D.M., Mcfarland, R., Taylor, R.W., and Thorburn, D.R. (2011). Respiratory chain complex I deficiency caused by mitochondrial DNA mutations. *Eur J Hum Genet* 19**,** 769-775.

Tang, Y., and Le, W. (2016). Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. *Mol Neurobiol* 53**,** 1181-1194.

Vasquez, O.L., Almeida, A., and Bolanos, J.P. (2001). Depletion of glutathione up-regulates mitochondrial complex I expression in glial cells. *J Neurochem* 76**,** 1593-1596.

Viader, A., Sasaki, Y., Kim, S., Strickland, A., Workman, C.S., Yang, K., Gross, R.W., and Milbrandt, J. (2013). Aberrant Schwann cell lipid metabolism linked to mitochondrial deficits leads to axon degeneration and neuropathy. *Neuron* 77**,** 886-898.

Volobоueva, L.A., Emery, J.F., Sun, X., and Giffard, R.G. (2013). Inflammatory response of microglial BV-2 cells includes a glycolytic shift and is modulated by mitochondrial glucose-regulated protein 75/mortalin. *FEBS Lett* 587**,** 756-762.

Von Bartheld, C.S., Bahney, J., and Herculano-Houzel, S. (2016). The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting. *J Comp Neurol* 524**,** 3865-3895.

West, P. Mitochondrial Dysfunction as a Trigger of Innate Immune Responses and Inflammation. *This Special Issue*.

Wu, J., Holstein, J.D., Upadhyay, G., Lin, D.T., Conway, S., Muller, E., and Lechleiter, J.D. (2007). Purinergic receptor-stimulated IP3-mediated Ca2+ release enhances neuroprotection by increasing astrocyte mitochondrial metabolism during aging. *J Neurosci* 27**,** 6510-6520.

Ye, J., Jiang, Z., Chen, X., Liu, M., Li, J., and Liu, N. (2016). Electron transport chain inhibitors induce microglia activation through enhancing mitochondrial reactive oxygen species production. *Exp Cell Res* 340**,** 315-326.

Yin, F., Boveris, A., and Cadenas, E. (2014). Mitochondrial energy metabolism and redox signaling in brain aging and neurodegeneration. *Antioxid Redox Signal* 20**,** 353-371.

Yuan, Y.H., Sun, J.D., Wu, M.M., Hu, J.F., Peng, S.Y., and Chen, N.H. (2013). Rotenone could activate microglia through NFkappaB associated pathway. *Neurochem Res* 38**,** 1553-1560.

Zhang, X., Haaf, M., Todorich, B., Grosstephan, E., Schieremberg, H., Surguladze, N., and Connor, J.R. (2005). Cytokine toxicity to oligodendrocyte precursors is mediated by iron. *Glia* 52**,** 199-208.

CHAPTER 3

MITOCHONDRIAL METABOLISM IN ASTROCYTES REGULATES BRAIN BIOENERGETICS, NEUROTRANSMISSION AND REDOX BALANCE

3.1 Introduction

The central nervous system (CNS) is a complex, integrated structure composed of diverse cell types. Neurons are highly specialized cells in charge of transmitting, processing, and storing information and thus have been considered the functional units of the CNS. However, research within the last few decades has demonstrated critical roles non-neuronal cells play in CNS function. The non-neuronal cells of the CNS include macroglia (astrocytes, ependymal cells and oligodendrocytes) and microglia, which support essential functions such as the maintenance of neurotransmitter communication, metabolism, trophic support, formation of myelin sheath, wound healing, and immune surveillance (von Bernhardi et al., 2016) .

Astrocytes originate from the neuroectoderm. Recent estimates suggest they account for between 19-40% of the glial population (with an estimated glial to neuron ratio of ~1:1), and a strong regional variation within the CNS (von Bartheld et al., 2016). Astrocytes are a heterogenous population of cells with a diverse array of morphological, functional and molecular properties (Garcia-Marin et al., 2007). While the cellular identity of astrocytes is determined by their gene expression profile post-differentiation (intrinsic patterning) (Song et al., 2002;Krencik et al., 2011), the surrounding environment and neuronal circuitry further shapes, modifies, and maintains the form and functions of differentiated astrocytes (extrinsic factors) (Koulakoff et al., 2008;Yang et al., 2009;Farmer et al., 2016;Ben Haim and Rowitch, 2017). Structurally, astrocytes contain multiple radial processes that create diverse interfaces with other glia, neurons, and capillary endothelial cells. Astrocytes' fine processes and endfeet envelope neuronal cell bodies,

synapses, and blood vessels. In addition, an intracellular interface with other astrocytes or oligodendrocytes is generated via gap junctions (connexin channels), creating an extensive interconnected network throughout the brain (Orthmann-Murphy et al., 2008). Gap junctions exchange ions and small molecules between cells (<1kDa in size) including; inositol triphosphate (IP_3), K^+, Ca^{2+}, ATP, glucose, glutamate, glutathione (GSH), and cyclic AMP; highlighting its signaling and buffering role within the CNS (Niessen et al., 2000;Goldberg et al., 2002;Bedner et al., 2006;Lapato and Tiwari-Woodruff, 2018).

Astrocytes are involved in a number of processes associated with brain function and disease progression. Astrocytes are major secretory cells, releasing factors or transmitters (gliotransmitters) including neurotransmitters (and their precursors), modulators, peptides, hormones, trophic (growth) factors, and metabolites. Astrocyte secreted factors have been shown to be involved in synapse formation, function and plasticity, neuronal growth, differentiation and survival, as well as in the regulation of the vascular tone and blood flow in the brain. Astrocytes have also been shown to mediate synaptic pruning by phagocytosis (Chung et al., 2015;MacVicar and Newman, 2015;Verkhratsky et al., 2016).

Astrocytes become “reactive” as a response to pathological conditions or to perturbations in cellular homeostasis. Astrocyte reactivity is a loose term that primarily refers to an enlargement in the cell body and processes (hypertrophy), and an increase in the levels of the glial fibrillary acidic protein (GFAP), that relates to a pathological stimulus in the CNS. Unlike microglia, reactive astrocytes are not believed to be proliferative outside of conditions of glial scar formation, which is a specific, irreversible form of astrocyte reactivity. Reactive astrocytes contribute to the production and release of cytokines and the neuroinflammatory process during injury and neurodegeneration. Reactive astrocytes should not be confused with activated

astrocytes, as the latter pertains to astrocyte responses to neurotransmission (Ben Haim et al., 2015).

Astrocytes can also be considered the master regulators of brain metabolism. In this thesis chapter, the importance of astrocytes' metabolism in the regulation of neurotransmitter recycling and synthesis, central carbon metabolism and bioenergetics, as well as redox homeostasis and antioxidant/xenobiotic defense is discussed. Our goal is to highlight the metabolic niche astrocytes fill within overall brain metabolism.

3.2 Astrocytes' metabolism and bioenergetics: a glycolytic cell

While the mass of the human brain accounts for approximately 2% of total body weight, its oxidative metabolism accounts for ~20% of the body's total oxidative metabolism (Rolfe and Brown, 1997;Attwell and Laughlin, 2001). Most of this oxidative energy (estimated 75-80%) is utilized at the synapse to maintain and restore ionic gradients, and for the uptake and recycling of neurotransmitters (Riveros et al., 1986;Wong-Riley, 1989;Attwell and Laughlin, 2001;Hyder et al., 2013) **(Figure 3.3.4)**. By comparison, the metabolic processes in astrocytes have been estimated to account for between 5% and 15% of the total ATP expenditure in the brain (Attwell and Laughlin, 2001;Belanger et al., 2011). Glucose is the primary energy substrate in the adult brain, however alternate energy sources can fuel brain function. Lactate transport across the blood-brain barrier (BBB) **(Figure 3.3.1)** provides ~8%–10% of the brain's energy requirements under basal conditions (Boumezbeur et al., 2010), and it has been estimated to supply ~20%–25% of energy during energy demanding activities (van Hall et al., 2009). As plasma lactate concentrations rise, lactate uptake in the CNS increases coinciding with a decrease in

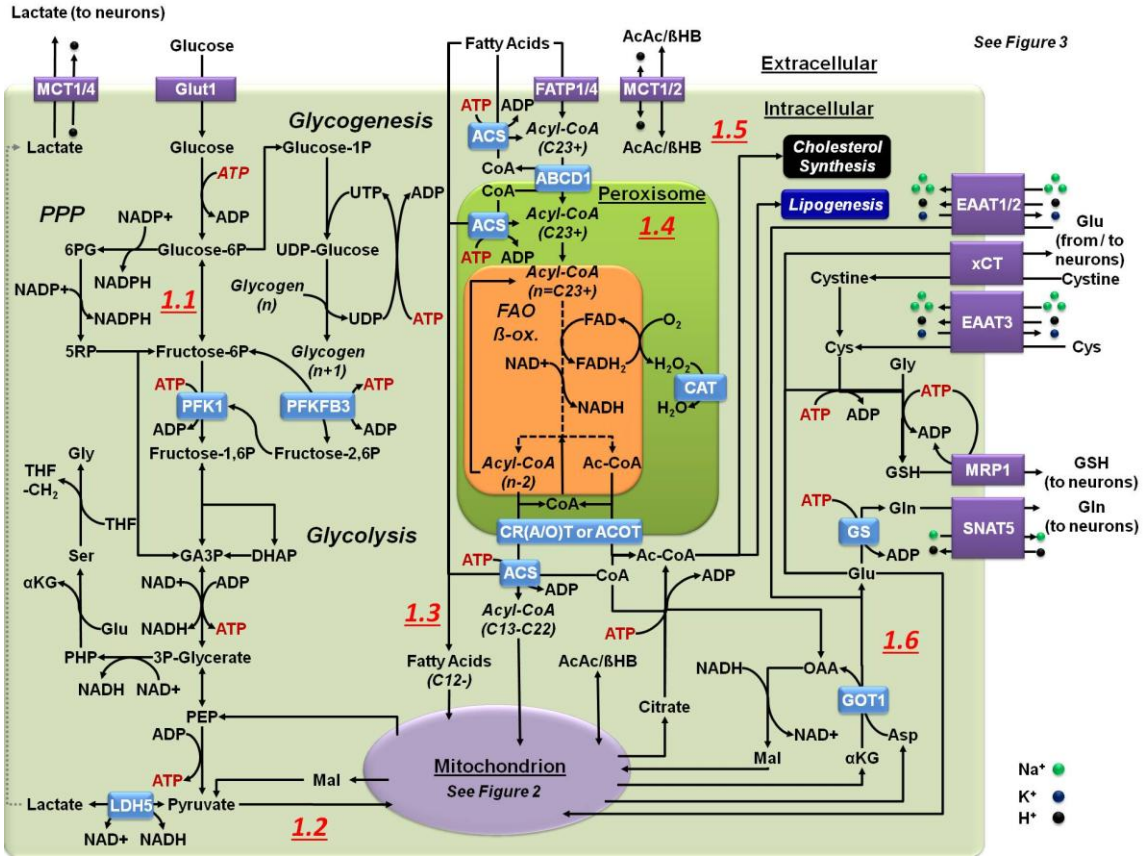


Figure 3.1: Central Carbon Metabolism in Astrocytes

(1.1) In astrocytes, glucose is used via glycolysis as a principal energy source. The PPP creates redox potential by replenishing the NADPH pool. Glucose storage in glycogen is restricted or transient at most. Glucose carbons can also be used for cysteine (Cys) (trans-sulfuration, *not shown*) and glycine (Gly) synthesis.

(1.2) Pyruvate is the principal end product of glycolysis in astrocytes. Astrocytes convert most pyruvate into lactate by LDH5 to be export to the extracellular space. Pyruvate also enters the mitochondria (see Figure 2 for details).

(Figure 3.1: Central Carbon Metabolism in Astrocytes continued)

(1.3) FAs are sorted by their size: 12 or less carbons can directly enter the mitochondria, 13-22 carbons pass into the mitochondria through a shuttle (see Figure 2 for details), and 23+ carbons enter peroxisomes to create shorter FA products through α/β-oxidation. The esterification of FAs (ACS) limits their ability to pass through membranes, and is required for FAO.

(1.4) Peroxisomal α/β-oxidation occurs in a similar manner to mitochondrial FAO but, without an ETC, $FADH_2$ donates its electrons directly to oxygen to create H_2O_2 that is neutralized by CAT. The fate of NADH in peroxisomes has yet to be determined. Multiple species of FA transport systems exist within peroxisomes, each with different affinities for various FA lengths. Exported FAs enter the mitochondria to complete FAO.

(1.5) Ketone bodies in astrocytes can enter as substrates for mitochondrial metabolism (see Figure 2 for details) or can be the end product in mitochondrial metabolism that astrocytes export for other cells.

(1.6) Astrocytes utilize glutamate (Glu) for glutamine (Gln) synthesis (Glu-Gln cycle), Cys acquisition (xCT or EAAT3), and for GSH synthesis, this later exported via (MRP1). In astrocytes, Glu is imported from the synaptic cleft by EAAT1/2 or derived from transamination of the mitochondrial TCA intermediate αKG by Asp amino transferases (GOT1/2). Glu can also be used to power the malate (Mal)-Asp shuttle through AGC or Aralar 1, or enter the mitochondria as a substrate for oxidative phosphorylation (see Figure 2 for details).

glucose uptake (Smith et al., 2003;van Hall et al., 2009). When required, an important pool of lactate is always available as the extracellular levels of lactate (0.5-1.5 mM) are similar to those of glucose (Magistretti and Allaman, 2015).

Metabolism in astrocytes and neurons is interconnected, and it has been clearly demonstrated that neurons depend upon astrocytes for a variety of metabolic processes. The *in vivo* energy demands of astrocytes can be fulfilled in the absence of oxidative phosphorylation, with an observed increase in lactate production in mutants of *Cox10* (part of the cytochrome c oxidase complex and required for complex IV function), indicating that astrocytes are glycolytic **(Figure 3.1.1** and **3.3.2)** (Supplie et al., 2017). Glucose has been reported as being the preferential energy substrate of astrocytes over lactate, though glucose uptake does not match theoretical bioenergetic output (Bouzier-Sore et al., 2006;Jakoby et al., 2014). Pyruvate entry into the tricarboxylic acid (TCA or Krebs) cycle **(Figure 3.2.1)** is limited by a reduced activity of pyruvate dehydrogenase (PDH) in astrocytes due to phosphorylation (Bouzier-Sore et al., 2006;Halim et al., 2010;Jakoby et al., 2014) creating a surplus of pyruvate due to the high levels of glucose consumption **(Figure 3.1.2)**. In contrast to astrocytes, glycolysis in neurons is restricted by the continuous degradation of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase-3 (PFKFB3), which catalyzes the synthesis of fructose-2,6-bisphosphate (F2,6BP) an allosteric activator of phosphofructokinase 1 (PFK1) (Almeida et al., 2004;Herrero-Mendez et al., 2009). Accordingly, glucose utilization in neurons is directed to the pentose phosphate pathway (PPP) in order to regenerate nicotinamide adenine dinucleotide phosphate (NADPH) for the reductive recycling for GSH upon oxidative stress **(Figure 3.3.3)** (Delgado-Esteban et al., 2000;Vaughn and Deshmukh, 2008;Herrero-Mendez et al., 2009;Bolanos, 2016). The astrocyte-neuron lactate shuttle (ANLS) hypothesis states that a large portion of glucose metabolism in astrocytes is directed to lactate production and this is subsequently shuttled to

neurons as fuel for oxidative phosphorylation (OXPHOS) **(Figure 3.3.2** and **3.3.3)** (Pellerin and Magistretti, 1994;Magistretti and Allaman, 2015;Machler et al., 2016). However, recent studies also demonstrate that during energy-demanding conditions neurons have the capacity to upregulate glucose metabolism, and have been used to argue against the ANLS hypothesis **(Figure 3.3.3)** (Patel et al., 2014;Lundgaard et al., 2015;Diaz-Garcia et al., 2017).

Astrocytes also store energy in the form of glycogen, which can be broken down to lactate. Yet due to the limited amount of glycogen stored in astrocytes (~3 to 12 μmol glycogen per g of tissue, 1-6% of total glucose (Choi et al., 1999)), its contribution to the maintenance of either neuronal or astrocyte energy-dependent processes under stress conditions is still unclear (Bak et al., 2018). There is strong evidence supporting a robust oxidative metabolic capacity in astrocytes (1/3 of total brain glucose oxidative metabolism and 1/2 of total brain lactate oxidative metabolism) (Zielke et al., 2009), and even higher transcriptional levels of both glycolytic and TCA cycle enzymes in astrocytes *vs* neurons, as well as a high concentration of mitochondria in astrocyte endfeet **(Figure 3.3.6)** (Lovatt et al., 2007). Nevertheless, astrocytes deficient in mitochondrial respiration survive as glycolytic cells, both *in vitro* and *in vivo* (Supplie et al., 2017). What then is the physiological importance of mitochondrial metabolism in astrocytes? We next discuss the particularities of mitochondrial metabolism in these glial cells and its importance for neurotransmission and redox balance.

3.3 Mitochondrial metabolism in astrocytes: Anaplerosis and Cataplerosis

Oxidative metabolism in mitochondria is primarily fueled by pyruvate, fatty acids/ketone bodies, and glutamine/glutamate. Pyruvate generated from glycolysis is actively transported into the mitochondrial matrix via the mitochondrial pyruvate carrier (MPC1) to be subsequently decarboxylated and combined with coenzyme A (CoA) **(Figure 3.2.1)**. Oxidative decarboxylation

of pyruvate to acetyl-CoA is mediated by PDH and is coupled with the reduction of NAD^+ to NADH. Subsequently, citrate synthase catalyzes the condensation of oxaloacetate with acetyl-CoA to generate citrate. Pyruvate decarboxylation is the primary carbon source for the TCA cycle and is sufficient to sustain oxidative carbon flux during energy consumption, as the last step in the cycle regenerates oxaloacetate to be combined further with another acetyl-CoA molecule generated by PDH (a closed loop) **(Figure 3.2.1)**. However, TCA cycle intermediates also provide precursors for the biosynthesis of several classes of molecules. TCA intermediates leave the cycle via cataplerotic reactions linked to biosynthetic processes, while anaplerotic reactions supplement carbons back into to the TCA cycle. A balance between anaplerosis and cataplerosis controls the entry and exit of carbons into the TCA cycle, and is essential for biosynthetic processes **(Figure 3.2.1)** (Owen et al., 2002).

Pyruvate carboxylase (PC) is a major anaplerotic enzyme in the mitochondria that generates oxaloacetate from pyruvate in an ATP-dependent manner (**Figure 3.2.1**), replenishing carbons lost by the export of oxaloacetate, citrate, and α-ketoglutarate (αKG) from the TCA cycle, for gluconeogenesis, fatty acid (FA) synthesis, and amino acid synthesis, respectively **(Figure 3.1.2, 3.1.6** and **3.2.6)**. In astrocyte gluconeogenesis, oxaloacetate is converted to phosphoenolpyruvate (PEP) in the mitochondria by phosphoenolpyruvate carboxykinase (PEPCK2), as cytosolic phosphoenolpyruvate carboxykinase (PEPCK1) activity has not been detected in the astrocyte cytosolic fractions (Schmoll et al., 1995). While PEP has been shown to exit mitochondria (Garber and Ballard, 1969;Stark et al., 2009), the precise mechanism has not been determined, with the citrate isocitrate carrier (CIC) and adenine nucleotide transporter (ANT) being proposed (Stark and Kibbey, 2014). In the brain, it has been long recognized that PC is primarily expressed in astrocytes and to a lesser extent in microglia, oligodendrocytes and ependymal cells (Shank et al., 1985;Murin et al., 2009). However, as mentioned above, PDH

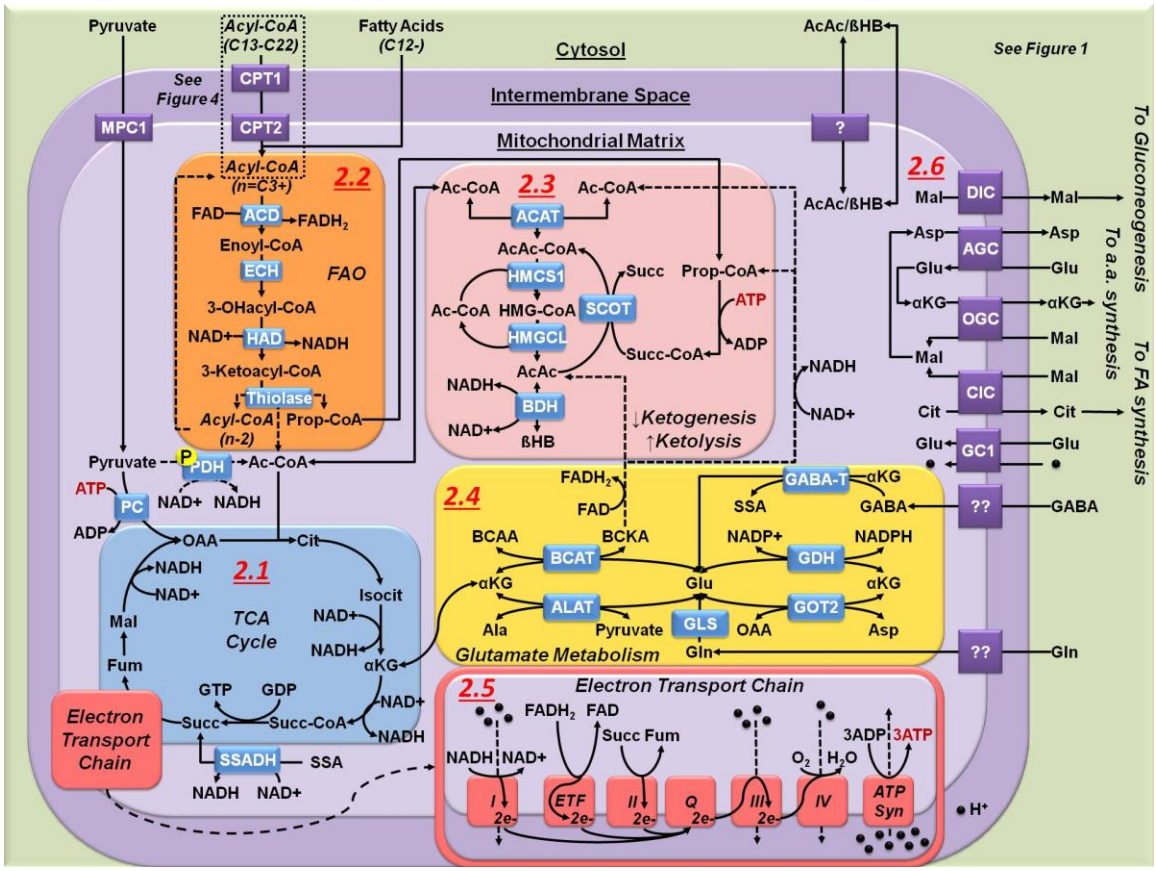


Figure 3.2: Mitochondrial Metabolism in Astrocytes

(2.1) The astrocyte TCA cycle is principally used for biosynthetic processes, not OXPHOS. Synthesis of Ac-CoA from pyruvate is limited by the phosphorylation of PDH (P) Cataplerotic reactions of the TCA cycle are matched by anaplerotic carbon inputs. Anaplerotic inputs in astrocyte include pyruvate carboxylatyion to OAA, acetyl-CoA (Ac-CoA) and succinyl-CoA (succ-CoA) synthesis from FAO, and ketolysis Ac-CoA and glutamate, and GABA neurotransmitter synthesis from αKG and succinate, respectively. Astrocyte cataplerotic outputs include αKG, malate, and citrate.

(2.2) FAO produces Ac-CoA and propionyl-CoA (Prop-CoA) that are directed to either ketogenesis, ketolysis, or as anaplerotic input to the TCA cycle. FAO generates a high yield of NADH and $FADH_2$ (*see 2.5*).

(Figure 3.2: Mitochondrial Metabolism in Astrocytes continued)

(2.3) Ketogenesis condenses Ac-CoA to either acetoacetate (AcAc) or β-hydroxybutyrate (ßHB). FAO is the principal source of ketogenesis in astrocytes. Ketolysis reverses this process, requiring succ-CoA from either prop-CoA (FAO or BCAA metabolism [not shown]), or the TCA cycle as a driving force.

(2.4) Mitochondrial Glu synthesis and incorporation into the TCA cycle in astrocytes is performed by transamination (GOT2, ALAT, BCAT) or by deamination (GDH, GLS, GABA-T) of αKG allowing glutamate to act in both cataplerosis and anaplerosis.

(2.5) The astrocyte ETC is not required for survival, and its activity has been reported to be a source of ROS due to inefficient complex activity and electron transfer. Importantly high yield of NADH and $FADH_2$ generated from FAO (*see 2.2*) can increase ROS formation due to the transfer of electrons from $FADH_2$ to ETF, creating a backpressure of electrons flowing from complex I.

(2.6) While the outer mitochondrial membrane is fairly permeable, numerous transporters exist on the inner membrane to facilitate entry and exit of a number of metabolites from the mitochondrial matrix.

activity in astrocytes is reduced by its phosphorylation and thus an additional carbon source is proposed to exist to supplement acetyl-CoA to the TCA cycle **(Figure 3.2.1)** (Halim et al., 2010). FA oxidation (FAO) has been proposed to be the preferable substrate for acetyl-CoA synthesis in astrocytes **(Figure 3.1.4** and 3.**2.2)**, but the physiological implications are still unclear (Halim et al., 2010;Adina-Zada et al., 2012;Panov et al., 2014).

3.4 FA oxidation (FAO) in astrocytes: The "missing link"

Ketone bodies are water-soluble molecules derived from FA metabolism in the liver, supplying energy to peripheral tissues including the brain. Ketone bodies have been shown to be produced by astrocytes as well (Blazquez et al., 1998;Cullingford et al., 1998;Guzman and Blazquez, 2001;Thevenet et al., 2016). During starvation up to ~ 70% of the brain's energy can be supplied by ketone bodies (Owen et al., 1967). Ketone bodies are produced in the mitochondria from acetyl-CoA in three sequential steps mediated by: 1) acetyl-CoA acetyltransferase 1 (ACAT1) also known as acetoacetyl-CoA thiolase or thiolase II (acetyl-CoA → acetoacetyl-CoA, a reversible reaction); 2) β-hydroxy-β-methyl glutaryl (HMG)-CoA synthase (HMCS1, acetoacetyl-CoA → HMG-CoA); and 3) HMG-CoA lyase (HMGCL, HMG-CoA → Acetoacetate). Acetoacetate (AcAc) is then decarboxylated to acetone or metabolized to β-hydroxybutyrate (βHB) via β-hydroxybutyrate dehydrogenase (BDH) **(Figure 3.2.3)**. Ketone bodies can cross the BBB and plasma membranes via monocarboxylate transporters (MCT1, 2, 4) **(Figure 3.1.5, 3.3.1** and **3.3.4)** (Halestrap and Price, 1999;Morris, 2005). At high concentrations, ketone bodies have been proposed to support basal and activity-dependent needs of neurons and glutamatergic signaling, while decreasing glucose utilization (Lopes-Cardozo et al., 1986;Kunnecke et al., 1993;Sibson et al., 1998;Magistretti et al., 1999;Chowdhury et al., 2014;Courchesne-Loyer et al., 2017). Within the cell, ketone body transport across the

mitochondrial membrane has not been fully characterized. While the mitochondrial pyruvate carrier (MPC1) has been implicated in ketone body transport, inhibition of the MPC1 does not abolish ketone body accumulation in the mitochondria, indicating that an undetermined transporter system or diffusion is present **(Figure 3.2.6)** (Paradies and Papa, 1977;Halestrap, 1978;Booth and Clark, 1981;Achanta and Rae, 2017). In mitochondria, ketolysis starts when BDH converts β-hydroxybutyrate back to acetoacetate, which is activated to acetoacetyl-CoA by succinyl-CoA-3-oxoacid-CoA transferase (SCOT). Acetoacetyl-CoA is then degraded by ACAT1 generating acetyl-CoA that can be incorporated into the TCA cycle **(Figure 3.2.3)**. Neurons, astrocytes and oligodendroglia have been shown to be fully capable of using ketone bodies as metabolic fuel *in vitro* (Chechik et al., 1987).

In addition to ketone bodies, FAs are also energy substrates for the brain, and unlike ketone bodies, they diffuse across the BBB rather than relying on transport systems for their influx. The main source of FAs crossing into the brain are non-esterified long-chain FAs complexed with albumin and circulating lipoproteins (Hamilton and Brunaldi, 2007). Transport of non-esterified FAs across the BBB is mediated by passive diffusion (FA flip-flop) or by FA transporters including FA transport proteins (FATP1 and 4, with intrinsic Acyl-CoA synthetase activity) and the FA translocase (FAT/CD36) **(Figure 3.3.1)** (Gnaedinger et al., 1988;Purdon et al., 1997;Hamilton and Brunaldi, 2007;Ouellet et al., 2009;Mitchell et al., 2011). The exact transport mechanisms that mediate the uptake of FAs into neurons or astrocytes are still unclear. A recent report suggests that FATP1 and 4 mediate FA uptake in glial cells. Importantly, while neurons are capable of synthesizing significant amounts of lipids during bioenergetic excess, they are reported to be dependent on an apolipoprotein E (ApoE) shuttle system to transfer them to astrocytes to avoid neurodegenerative phenotypes **(Figure 3.3.6)** (Liu et al., 2015;Liu et al., 2017). To utilize FAs metabolically (FAO), FAs must be esterified to CoA in an ATP-dependent

process by acyl-CoA synthetases (ACS), a process that is often linked to, or performed shortly after, FA transport by FATPs **(Figure 3.1.3)** (Mashek et al., 2007;Anderson and Stahl, 2013). In the brain, signaling through peroxisome proliferation activated receptor beta (PPARß, predominantly expressed in neurons but also glia) increases the expression of ACS2 (Basu-Modak et al., 1999), in turn increasing the oxidation of exogenously supplied FAs in neurons (Marszalek et al., 2004). Astrocytes have an increased expression of both PPARα and ACS1 compared to neurons (Basu-Modak et al., 1999), which has been linked to the upregulation of genes involved in FAO and ketogenic processes (Cullingford et al., 2002). Carnitine palmitoyltransferase 1a (CPT1a) transfers the acyl groups from esterified FAs (acyl-CoA) to L-carnitine, producing acylcarnitine esters which are transported across the outer and inner mitochondrial membranes via the voltage-dependent anion channel (VDAC) and the carnitine/acylcarnitine translocase (CACT) transport, respectively. Then, CPT2 converts acylcarnitine back to acyl-CoA in the mitochondrial matrix **(Figure 3.3.4)** (Houten and Wanders, 2010;Panov et al., 2014;Romano et al., 2017).

In the mitochondria, acyl-CoAs are principally broken down into acetyl-CoA by β-oxidation in a series of sequential reactions that include: 1) an initial FAD-dependent dehydrogenation of acyl-CoAs by acyl-CoA dehydrogenase (ACD) that generates $FADH_2$; 2) a subsequent hydration step mediated by enoyl-CoA hydratase (ECH) forming 3-hydroxy(OH)acyl-CoA; 3) generation of 3-ketoacyl-CoA via 3-OHacyl-CoA dehydrogenase (HAD) in an NAD+-dependent manner regenerating NADH; and finally 4) cleavage and release of acetyl-CoA via thiolase I leaving an acyl-CoA two carbon atoms shorter that re-enters the pathway. The last three steps are carried by a heteroctamer protein complex called the mitochondrial trifunctional protein **(Figure 3.2.2)** (Houten and Wanders, 2010;Panov et al., 2014;Romano et al., 2017). For FAs/acyl-CoAs with odd numbered carbons, β-oxidation will

yield propionyl-CoA rather than acetyl-CoA from the final carbons of the acyl-CoA. Propionyl-CoA is converted to succinyl-CoA in an ATP-dependent manner by a series of mitochondrial enzymes, providing an anaplerotic input to the TCA cycle and acting as a co-substrate in the ketolytic conversion of acetoacetyl-CoA to acetoacetate by SCOT **(Figure 3.2.3)**. In the event that the β-carbon of a FA is methylated, α-oxidation occurs to remove one carbon unit as formic acid (formic acid is converted to carbon dioxide (CO_2)), shifting the methylated carbon from the β to α position, allowing β-oxidation to resume. The steps in α-oxidation include: 1) hydroxylation of the α-carbon by phytanoyl-CoA hydroxylase (PhyH/Pahx) utilizing the conversion of αKG to succinate as a driving force; 2) decarboxylation by 2-hydroxyphytanoyl-CoA lyase (2-HPCL) releasing formyl-CoA and a fatty aldehyde; 3) oxidation of the fatty aldehyde by aldehyde dehydrogenase generating NADH and an acyl-CoA one carbon shorter than the original product (Jansen and Wanders, 2006).

A large quantity of ATP can be generated from FAO (one molecule of palmitic acid provides approximately 115 ATPs vs 32 ATPs generated from glucose oxidation). Despite its high-energy potential, FAs have long been considered a poor energy substrate in the brain. The brain as a whole was thought to be limited in β-oxidation capacity as thiolase I, ACD and ECH activities are 0.7%, 50% and 19% that of the heart mitochondria (Yang et al., 1987), though it must be noted that glial and neuronal forms of the enzymes were not assayed independently **(Figure 3.2.2)**. While there are three known carnitine palmitoyltransferase 1 (CPT1) isoforms, CPT1a has a low expression in the whole brain and is not expressed in neurons (Jernberg et al., 2017), CTP1b is not expressed in the brain (Obici et al., 2003;Lavrentyev et al., 2004), and CPT1c localizes exclusively to the endoplasmic reticulum in neurons and has not been shown to participate in FAO (Wolfgang et al., 2006;Sierra et al., 2008) but has been implicated in ROS management (Lee and Wolfgang, 2012). Lower CPT1a expression and activity restricts

mitochondrial oxidation of FAs (Bird et al., 1985), except for those with 12 or fewer carbons, which can passively diffuse across the mitochondrial inner membrane **(Figure 3.1.3, 3.2.2)**. Yet the brain has been demonstrated to metabolize carbons from FAs *in vivo*, accounting for ~20% of the total acetyl-CoA pool when labeled octanoate is supplied to the bloodstream (Ebert et al., 2003).This is believed to occur predominantly in astrocytes, based on comparisons of astrocytes, oligodendrocytes and neurons in primary cultures (Edmond et al., 1987).

Astrocytes have a high buffering capacity against ROS compared to neurons (Sun et al., 2006), and FAO is proposed to induce higher amounts of ROS formation. One cycle of β-oxidation generates one molecule of $FADH_2$ and NADH, increasing the total $FADH_2$/NADH ratio per acetyl-CoA created compared to glycolysis **(Figure 3.2.2)**. When ACD transfers electrons from acyl-CoA to FAD, it is coupled to the subsequent transfer of electrons from the resultant $FADH_2$ to the electron transferring flavoprotein (ETF). The follow-up transfer of electrons from ETF to ubiquinone by ETF dehydrogenase creates a backpressure of electrons flowing through complex I (donated from NADH) and complex II (donated from succinate) to ubiquinone that enhances the probability of leakage and formation of ROS **(Figure 3.2.5)**. Thus, it has been proposed that restricting FAO protects neurons against oxidative damage beyond the stages of development (Schonfeld and Reiser, 2013;2017). In the brain, inhibition of FAO by methyl palmoxirate has been shown to reduce the concentrations of non-enzymatically oxidized metabolites derived from poly-unsaturated fatty acid (PUFA), indicating that ROS are being generated from FAO (Chen et al., 2014).

However, mechanism of ROS generation from FA substrates is not universal, and depends upon the length and structure of the FA. Very long chain FAs (VLCFAs) that are not esterified have been shown to integrate into artificial phospholipid bilayers, desorpting at slower

rates than shorter fatty acids and disrupting membrane fluidity (Ho et al., 1995). This integration and disruption by VLCFAs has been shown to occur in the inner mitochondrial membrane, decreasing the membrane potential by protonophoric activity, and reducing ROS generated from reverse electron transport (RET) (Hein et al., 2008). The protonophoric property, and its resulting decrease in ROS from hyperpolarization, has been demonstrated in medium chain FAs (MCFAs)(Korshunov et al., 1998), long chain FAs (LCFAs), and branched chain FAs (Schonfeld and Wojtczak, 2007). This protonophoric capacity of FAs decreases as the length of the FA decreases (Schonfeld and Wojtczak, 2016), while branched FAs such as phytanic acid show an increased protonophoric activity compared to their straight chain FA counterparts (Komen et al., 2007;Schonfeld and Reiser, 2016). Additionally, application of carnitine esters as substrates demonstrated that RET was not occurring in the mitochondria, furthering that RET is not a prominent form of ROS production during FAO (Schonfeld et al., 2010).

While protonophoric properties of FAs can mitigate the effects of hyperpolarization/RET, FAs can promote ROS generated through forward electron transport and by interfering with electron transport. Isolated mitochondria increased ROS production proportionally to FA exposure in the presence of the recoupling agent carboxyatractyloside (Schonfeld and Wojtczak, 2007) and during uncoupling with CCCP (Cocco et al., 1999), indicating that ROS was being generated independently of the protonophoric effects. Interestingly, branched phytanic acid and poly unsaturated arachidonic acid demonstrated stronger ROS effects than unbranched saturated FAs in the aforementioned studies (Cocco et al., 1999;Schonfeld and Wojtczak, 2007). Mitochondria exposed to low concentrations of palmitoylcarnitine were also found to generate ROS while maintaining membrane potential, with rotenone (complex I inhibition) having minimal effect on ROS while antimycin (complex III inhibition) dramatically increased ROS

(Seifert et al., 2010). As described in (Schonfeld and Wojtczak, 2008), despite the evidence of electron transfer interference by FAs, the mechanism is not well understood.

In the brain, FAO occurs primarily in astrocytes (Edmond et al., 1987;Auestad et al., 1991;Schonfeld and Reiser, 2013). While both astrocytes and oligodendrocytes uptake saturated and unsaturated FAs (Hofmann et al., 2017), CTP1a seems to be found primarily in astrocytes and neural progenitor cells, while absent in neurons, microglia, and oligodendrocytes (Jernberg et al., 2017). As such, carnitine deficiency causes a metabolic encephalopathy that is characterized by astrocytic swelling and mitochondrial expansion, corroborating that FAO is an essential metabolic component of astrocytic function (Kimura and Amemiya, 1990;Calabrese et al., 2005;Jones et al., 2010). CPT1a activity is negatively regulated by malonyl-CoA, a molecule synthesized by acetyl-CoA carboxylase (ACC) from cytosolic acetyl-CoA. Malonyl-CoA is also a principal component of fatty acid synthesis, making this molecule the regulator of FAO and lipogenesis (Foster, 2012). ACC is inhibited by phosphorylation via the energy sensor adenosine monophosphate (AMP)-activated protein kinase (AMPK), which links energy deficiency with mitochondrial β-oxidation (**Figure 3.3.4**). Recurrent low glucose exposure has been shown to activate AMPK and increase FAO dependency in astrocytes (Weightman Potter et al., 2019). In another study, astrocyte activation with ciliary neurotrophic factor (CNTF) was also shown to activate AMPK and increases β-oxidation and ketolysis (Escartin et al., 2007). In contrast, upon hypoxic conditions or high concentrations of FAs, ketogenesis is induced in astrocytes and this is regulated as well by AMPK signaling (Blazquez et al., 1999;Takahashi et al., 2014).

Peroxisomes are located throughout the brain but are primarily found in astrocytes and oligodendrocytes (Troffer-Charlier et al., 1998). FAs with more than 22 carbons cannot be transported into the mitochondria via the carnitine shuttle and thus, are broken down first by

peroxisomes. As in mitochondria, fatty acids must be esterified to CoA before undergoing FAO in peroxisomes. **(Figure 3.1.4** and **3.3.4)**. Because peroxisomes lack an ETC, electrons from $FADH_2$ are transferred directly to O_2 creating H_2O_2 that is scavenged by catalase (CAT). Within the peroxisome, FAs are shortened to acetyl-CoA, propionyl-CoA, and a wide range of long, medium, and short chain FAs (LCFAs/MCFAs/SCFAs) of 22 carbons or less. A variety of export systems exist within the peroxisomes, each with different affinities for different lengths of FAs, including carnitine-transferases (carnitine acetyltransferases (CRATs), carnitine octanoyltransferases (CROTs)), and thiolases (acyl-CoA thioesterases (ACOTs)) transport systems **(Figure 3.1.4)** (Antonenkov and Hiltunen, 2012). Additionally, peroxisomes have been shown to possess ketogenic enzymes, implicating peroxisomes as another potential source of ketone bodies and cholesterol (Hovik et al., 1991;Antonenkov et al., 2000;Olivier et al., 2000). Dysfunction of peroxisomal processes has been linked to demyelination, oxidative stress, inflammation, cell death, and abnormalities in neuronal migration and differentiation (Trompier et al., 2014). Defects in the ATP binding cassette subfamily D member 1 (ABCD1) transporter that translocates very LCFAs into peroxisomes **(Figure 3.1.4)**, leads to the accumulation of these fats in cells and tissues. VLCFAs and branched chain FAs can interfere directly with the ETC by opening the permeability transition pore, disrupting calcium balance and depolarizing the mitochondria (Hein et al., 2008;Kruska et al., 2015;Schonfeld and Reiser, 2016). LCFAs (13 to 21 carbons) activate peroxisome proliferator-activated receptors, transcription factors that stimulate both β-oxidation and ketogenesis as well as mitochondrial and peroxisome biogenesis. Recent reports have also demonstrated that peroxisome biogenesis requires the generation of pre-peroxisomes from mitochondrial derived vesicles (Sugiura et al., 2017).

3.5 Mitochondrial Metabolism in Astrocytes and Neurotransmitter Homeostasis

Astrocyte processes extending to synaptic clefts play an important role in regulating synaptic transmission, particularly glutamatergic signaling. After its vesicle-mediated release, the excitatory neurotransmitter glutamate is taken from the synaptic cleft by astrocytes in a Na^+ dependent manner via the excitatory amino acid transporters EAAT1 (glutamate aspartate transporter, GLAST) or EAAT2 (glutamate transporter 1, GLT-1) **(Figure 3.3.4)**. In the cytosol, glutamate is then converted into non-excitatory glutamine via glutamine synthetase (GS), and transferred back to neurons via the Na^+-coupled neutral amino acid transporters (SNATs) to be converted back into glutamate in neurons via phosphate-activated glutaminase (PAG) **(Figure 3.3.4)** (Norenberg and Martinez-Hernandez, 1979;Anderson and Swanson, 2000;Schousboe et al., 2013;Leke and Schousboe, 2016). In astrocytes, despite the presence of a weak mitochondrial targeting sequence, GS is localized in the cytosol because the mitochondrial membrane potential is not negative enough to drive GS into the mitochondria **(Figure 3.1.6** and **3.3.2)** (Matthews et al., 2010). Neurons lack PC activity (Yu et al., 1983), restricting their ability to replenish TCA intermediates lost during neurotransmitter release (anaplerotic reaction). Thus, the glutamate-glutamine cycle between neurons and astrocytes, not only terminates/modulates glutamatergic signaling, preventing excitotoxicity triggered by excessive or prolonged exposure to glutamate, but it also replenishes the neurotransmitter pool in neurons (Belanger et al., 2011) **(Figure 3.3.4)**.

Not all the glutamate taken from the synaptic cleft by astrocytes becomes glutamine. There is evidence that a significant portion is incorporated into the TCA cycle via oxidative metabolism **(Figure 3.2.4)**. Glutamate transport into the mitochondria is mediated via the aspartate/glutamate carrier antiporter (AGC or Aralar 1) or via the glutamate carrier (GC1 or SLC25A22), a symporter for glutamate and protons (H^+). Controversy still exists about the

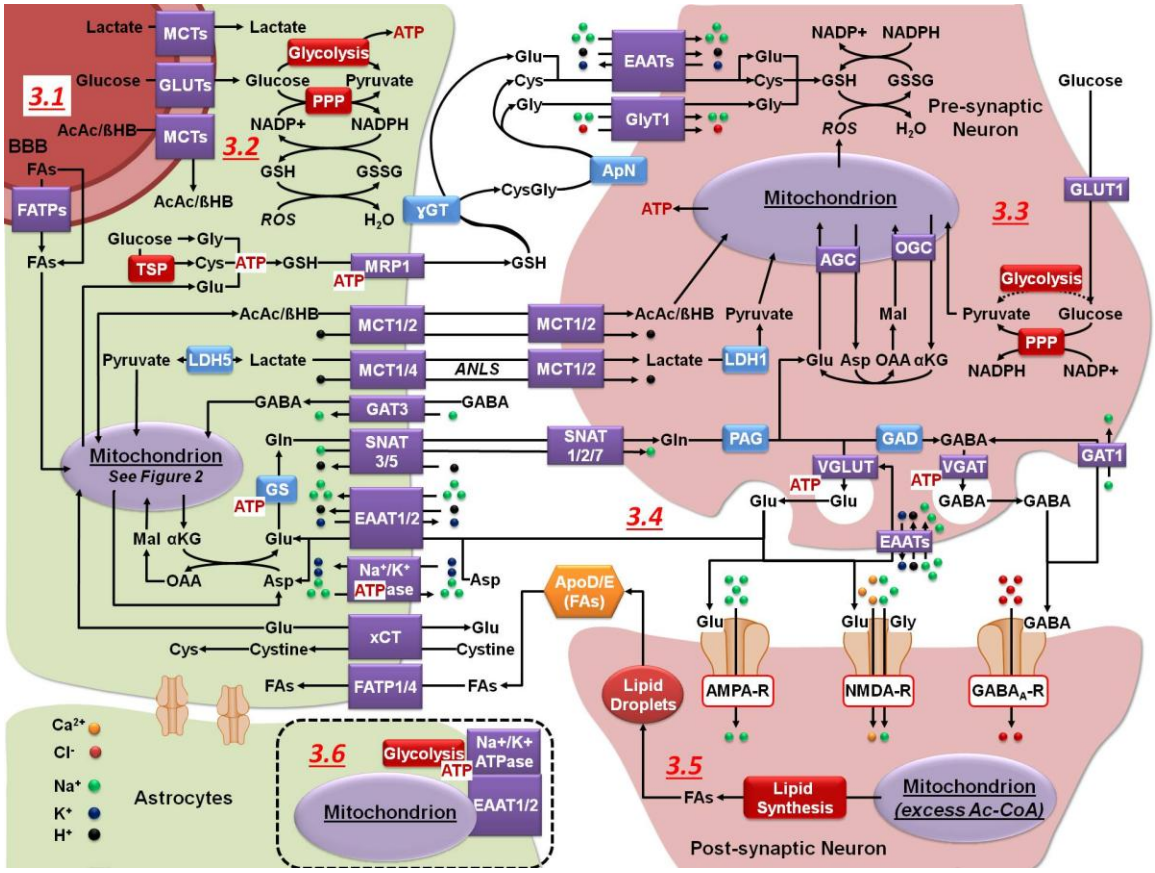


Figure 3.3: Neuronal-Astrocytic Metabolic Cooperation

(3.1) GLUTs (glucose), MCTs (Lactate and ketone bodies), and FATPs (fatty acids) transporters facilitate the transport of carbon sources across the BBB.

(3.2) Astrocytes utilize glucose for the production of ATP (glycolysis), regeneration of NADPH (PPP), and to feed mitochondrial biosynthetic processes. Astrocytes synthesize and export GSH (MRP1), lactate (MCT1/4), ketone bodies (MCT1/2), and Gln (at glutamatergic SNAT5 or GABAergic SNAT3 synapses) that can be utilized by neurons for energy production and synthesis of neurotransmitters.

(3.3) Neurons utilize glucose primarily for the regeneration of NADPH (PPP), with some studies suggesting that glycolysis can be upregulated during energy-demanding conditions. Lactate and ketone bodies are used in ATP production (OXPHOS). Neurons import GSH precursors from

(Figure 3.3: Neuronal-Astrocytic Metabolic Cooperation continued)

astrocytes via EAATs and GlyT1, lactate and ketone bodies via MCT1/2, and Gln through SNAT2/7 (SNAT1/7 in GABAergic neurons).

(3.4) Upon their release into the synaptic cleft Glu and GABA neurotransmitters can be uptaken into neurons via EAATs and the GABA transporter 1 (GAT1). However, astrocytes have a higher capacity to uptake and metabolize these neurotransmitters. In astrocytes, GABA is metabolized in the mitochondria (*see Figure 2.4*). Glu is taken in by astrocytes (EAAT1/2), converted to Gln (GS), and exported (SNAT5) for neuronal uptake (SNAT7). In the neurons, Gln is converted to Glu via PAG and then Glu can be converted to GABA via glutamate decarboxylase (GAD). Neurotransmitters are subsequently loaded into vesicles via vesicular transporters (VGLUT or VGAT), which do not require ATP *per se*, but their activity is coupled to that of the vacuolar H^+ ATPase. Na^+/K^+ ATPases maintain the electrochemical gradient required to maintain neuronal excitability (not shown) and in astrocytes, essential to drive neurotransmitter uptake.

(3.5) During periods of excess Ac-CoA lipid synthesis is initiated and transferred into lipid droplets. Lipids are then transported to astrocytes in an apolipoprotein D/E dependent manner, and taken in by fatty acid transporter proteins 1 and 4 (FATP1 and 4).

(3.6) In astrocytes, mitochondria co-localize on the plasma membrane with EAAT1/2, Na^+/K^+ ATPases, and glycolytic enzymes.

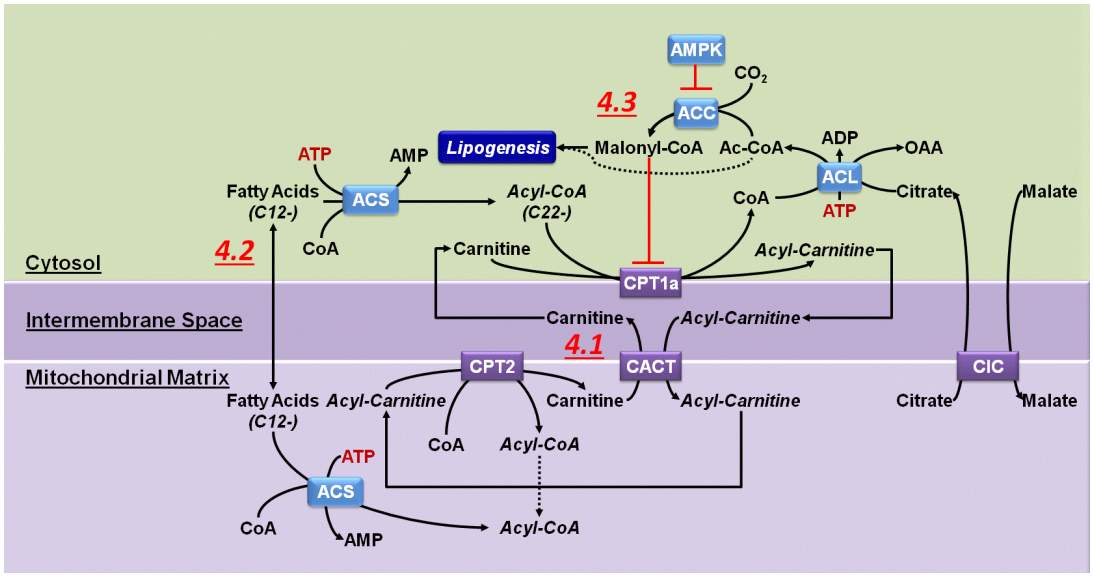


Figure 3.4: Fatty Acid Transport into the Mitochondria

(4.1) Coenzyme A thioesters (Acyl-CoAs) cannot cross mitochondrial membranes. CPT1a facilitates the transfer of the acyl group from CoA to carnitine. Acyl-carnitines are permeable to the outer mitochondrial membrane, but not the inner mitochondrial membrane, requiring the transport via carnitine-acylcarnitine translocase (CACT) to reach the mitochondrial matrix. Once in the matrix, CPT2 converts the acyl-carnitine back to an acyl-CoA, which can then undergo FAO (see Figure 2.2). The released carnitine is then transported back across the inner mitochondrial membrane by CACT to be reused in the cytosol.

(4.2) Non-esterified fatty acids (NEFAs) of 12 or less carbons do not require the carnitine shuttle system, and can diffuse across both mitochondrial membranes. However, they still require esterification to CoA by mitochondrial ACS before they can be metabolized by FAO.

(4.3) The carnitine shuttle system is regulated by malonyl-CoA, a precursor to fatty acid synthesis which also inhibits CPT1a activity. Malonyl-CoA is principally produced from citrate that is broken into OAA and acetyl-CoA in the cytosol by ATP citrate lyase (ACL). The released acetyl-CoA is carboxylated by acetyl-CoA carboxylase (ACC) to malonyl-CoA. ACC is modulated by AMPK activity, phosphorylating ACC and reducing ACC activity.

contribution of AGC to the uptake of glutamate in astrocytic mitochondria, as it has been shown to be primarily expressed in neurons. However, while the AGC activity in astrocytes corresponds only to 7% of the total brain AGC activity, it is calculated to be twice the minimum required for glutamate production and degradation **(Figure 3.2.6)** (Hertz, 2011). AGC is also central to the malate-aspartate shuttle (**Figure 3.1.6**), a system that allows the transfer of NADH electrons generated in the cytosol, from processes such as glycolysis, to the mitochondria (Amoedo et al., 2016). On the other hand, knockdown of GC1 suggests that this carrier is the main gate for net glutamate entry into mitochondria for oxidative metabolism in astrocytes **(Figure 3.2.6)** (Goubert et al., 2017). In the mitochondria, glutamate is metabolized to αKG by transamination via aspartate aminotransferase (GOT2), and to a lesser extent by alanine (ALAT) and branched chain aminotransferases (BCAT) **(Figure 3.2.4)**. In addition, α-KG can also be synthesized from glutamate via glutamate dehydrogenase (GDH), which is also a source of NADPH. While glutamate transamination via GOT2 generates a truncated TCA cycle due to the requirement of oxaloacetate as a co-substrate, GDH-mediated dehydrogenation can serve as an anaplerotic reaction to replenish TCA intermediates (**Figure 3.2.1** and **3.2.4)** (Sonnewald et al., 1993;Olsen and Sonnewald, 2015;McKenna et al., 2016). Astrocytes deficient in GDH increase their reliance on GOT2 and their glycolytic input to PC, which is paralleled by an accumulation of intracellular glutamate, underlying the importance of GDH in anaplerosis (Nissen et al., 2015;Pajecka et al., 2015). It should be noted that humans, unlike most other animals including mice and rats, have an additional GDH2 isoform that is expressed solely in astrocytes. Expression of GDH2 in rat cortical astrocytes increased glutamate uptake and metabolism, reducing their dependence on glucose and increasing their capacity to utilize branched chain amino acids (BCAA) (Nissen et al., 2017). Importantly, glutamate to glutamine metabolism is not determined by the extracellular

glutamate concentration. However, the entry of glutamate into the TCA cycle seems to be triggered by high extracellular glutamate concentrations (high μM) (McKenna et al., 1996).

The homeostasis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) is similarly linked to the glutamate-glutamine cycle. GABA is synthesized in neurons from the decarboxylation of glutamate by glutamate decarboxylase (GAD). After its vesicular release, GABA is uptaken by astrocytes through the GABA transporter (GAT3) **(Figure 3.3.4)**. GABA is then metabolized by GABA transaminase (GABA-T) with α-KG as cofactor to produce succinate semialdehyde (SSA) and glutamate **(Figure 3.2.4)**. SSA is metabolized to succinate via SSA-dehydrogenase (SSADH), which can then be incorporated back into the TCA cycle. This anaplerotic addition of succinate compensates the cataplerotic loss of carbons from the generation of glutamate by the GABA-T **(Figure 3.2.1)** (Schousboe et al., 2013).

In addition to metabolism, evidence indicates that astrocyte mitochondrial bioenergetics are important for the regulation of neurotransmitter balance. Both glutamate and GABA uptake systems in astrocytes are driven by Na^+ gradients that are established by the electrogenic action of the sodium-potassium adenosine triphosphatase (Na^+/K^+ ATPase) **(Figure 3.3.4)**. PC and GS activities also require ATP as well, and are critical enzymes in replenishing neuronal glutamine/glutamate **(Figure 3.2.1** and **3.3.4)**. Importantly, it has been demonstrated that mitochondria co-localize with EAAT1/2 on the plasma membrane of astrocytes as well as with glycolysis enzymes (Genda et al., 2011;Bauer et al., 2012) and glutamate transporters physically interact with the Na^+/K^+ ATPase **(Figure 3.3.6)** (Rose et al., 2009), suggesting that while mitochondrial-derived ATP is not required for astrocyte survival (Supplie et al., 2017), functional mitochondrial metabolism is important for neurotransmitter uptake and homeostasis **(Figure 3.3.2, 3.3.4** and **3.3.6)**. In fact, astrocyte processes enveloping synaptic terminals contain

abundant mitochondria (Jackson et al., 2014;Stephen et al., 2015;Jackson and Robinson, 2018), and inhibition of mitochondrial function by fluorocitrate in astrocytes increases glutamate excitotoxicity in co-cultures with neurons (Voloboueva et al., 2007).

3.6 Mitochondrial Metabolism in Astrocytes and Redox balance

Astrocytes have less efficient mitochondrial respiration and increased ROS formation when compared to neurons, in part due to a lower incorporation of complex I into supercomplexes in astrocytes caused by lower expression of the NADH-ubiquinone oxidoreductase core subunit S1 (NDUFS1) (Lopez-Fabuel et al., 2016). Regardless, scientific literature indicates that neurons have limited defense mechanisms against ROS compared to astrocytes. While both neurons and astrocytes can synthesize GSH, astrocytes have been reported to protect neurons via regulation of GSH metabolism (Chen et al., 2001;Shih et al., 2003). This protective effect is thought to be dependent on the supply of cysteine from astrocytes as a precursor for *de novo* GSH synthesis (Dringen et al., 1999;Wang and Cynader, 2000), and in part due to neurons being unable to uptake significant extracellular cystine (Sagara et al., 1993;Kranich et al., 1996). GSH has been proposed to be released from astrocytes via the ATP-binding cassette transporters subfamily C member 1 transporter (ABCC1, or multidrug-resistance-associated protein 1 [MRP1]) **(Figure 3.1.6** and **3.3.2)** (Hirrlinger et al., 2002;Hirrlinger and Dringen, 2005). Indeed, inhibition of MRP1 has been reported to reduce the extracellular accumulation of GSH in cultured astrocytes (Hirrlinger and Dringen, 2005). We have failed to observe a reciprocal accumulation of GSH in astrocytes when MRP1 activity is reduced (*unpublished data*), but this effect might be ascribed to feedback inhibition of GSH synthesis by GSH.

Once released, GSH is degraded by the γ-glutamyl transpeptidase (γGT) to produce l-cysteine-l-glycine (CysGly) (Dringen et al., 1997). CysGly is cleaved further by the neuronal aminopeptidase N (ApN) into the amino acids glycine and cysteine that are taken up by neurons for *de novo* GSH synthesis via GlyT1 and EAAT3, respectively **(Figure 3.3.2** and **3.3.3)** (Dringen et al., 1999;Dringen et al., 2001;Aoyama et al., 2008;Belanger et al., 2011). The export of GSH should imply a loss of carbons that must be replenished in astrocytes to maintain cellular redox homeostasis. Cysteine acquisition in astrocytes is mediated by Na^+-dependent and -independent uptake systems, but the exact contribution of X_{AG}-/EAAT3 and X_c-/xCT systems is still under debate (Shanker et al., 2001;Seib et al., 2011). *De novo* synthesis via the trans-sulfuration (TSP) pathway is also an important source for cysteine in astrocytes, and was shown to be upregulated and utilized during oxidative stress **(Figure 3.3.2)** (Vitvitsky et al., 2006). In most cells, cysteine is considered the limiting substrate for GSH synthesis. However, because glutamate in astrocytes is also metabolized to glutamine, it might also be considered a limiting factor **(Figure 3.1.6)**. Because glutamate synthesis depends on mitochondrial anaplerotic metabolism, mitochondrial function might also be essential for GSH homeostasis **(Figure 3.2.1** and **3.2.4)**. Interestingly, GSH depletion upregulates mitochondrial activity and expression of complex I in astrocytes (Vasquez et al., 2001), but the exact mechanisms that regulate this phenomenon are still unclear.

3.7 Conclusions and perspectives

The scientific process has been aptly described as blind people attempting to describe an elephant while only focusing on particular parts of the animal. Each person is contextually correct, but only when the data is combined can we achieve an accurate understanding of the whole. While there are far more parts of the proverbial elephant to explore to complete the

picture, the recent evidence indicates that we must adjust our current understanding of brain metabolism, particularly as it pertains to astrocytes and neurons.

Current research indicates that metabolism in the brain extends far beyond glucose. Fatty acids, and their derived ketone bodies, are important sources of bioenergetic potential for the brain, particularly during development, as shown by the experiments *in vivo* and *in vitro* that follow its oxidation and integration into biomolecules. Glutamate is more than just a neurotransmitter kept in strict stoichiometry by the glutamate-glutamine cycle; it is a resource the brain uses for amino acid synthesis by transamination, for redox balance by generation of NADPH, and for anaplerosis to maintain the TCA cycle.

The brain is far from a homogeneous body of cells, and the literature paints a clear picture of metabolic specialization and intracellular cooperation/dependence. In this chapter, we have centered our attention to astrocytic mitochondrial metabolism and its role in the regulation of brain bioenergetics, neurotransmission and redox balance via neuronal-astrocyte metabolic interactions. Astrocytes have been demonstrated to be functional without OXPHOS *in vivo*, to provide a GSH-dependent protective effect to neurons, to function as neutralizing and cycling agents of glutamatergic/GABAergic signaling, and to be synthesizers/exporters of OXPHOS bioenergetic molecules (lactate/ketone bodies). In contrast, neurons have a metabolic specialization towards OXPHOS, spurning FAO, restricting glucose oxidation while maintaining the PPP for redox homeostasis, and limiting their ability to conduct mitochondrial biosynthesis due to a lack of pyruvate carboxylase for anaplerosis. Together, neurons shoulder the bioenergetic burden of neurotransmission by controlled alterations in cell potential, while astrocytes conduct biosynthesis and high ROS oxidative processes to supplement neuronal functions.

The key organelle in this differential specialization is the mitochondria. While the broad mitochondrial role in OXPHOS is well known in mammalian cells, specialization is evident within neurons and astrocytes. Astrocytes restrict the conversion of pyruvate to acetyl-CoA by their phosphorylation of PDH, limiting their utilization of glucose for OXPHOS but instead directing these carbons to biosynthesis through the TCA cycle by PC. Additionally, the efficiency of the astrocyte ETC is far less than neurons, resulting in higher ROS generation in astrocyte compared to neurons. Astrocytic mitochondria have been demonstrated to utilize FAO for the production of ketone bodies, a process that has not been shown to be significant in neurons. Further, evidence shows that astrocytes localize mitochondria to endfeet processes in conjunction with glutamate transporters and enzymes related to glycolysis. Coupled with the evidence that OXPHOS is not required in astrocytes in vivo, this paints a unique picture for astrocyte mitochondria compared to the prototypical mammalian cell. It is a mitochondria specializing in synthesis at the expense of OXPHOS, while the neuronal mitochondria specialize in OXPHOS at the expense of synthesis.

3.8 List of Abbreviations

2-HPCL	2-hydroxyphytanoyl-CoA lyase
3-OHacyl-CoA	3-hydroxyacyl-Coenzyme A
ABCC1/MRP1	adenosine-triphosphate-binding cassette transporter subfamily C member 1 or multidrug-resistance-associated protein 1
ABCD1	adenosine triphosphate binding cassette subfamily D member 1
AcAc	Acetoacetate
ACAT	acetyl-CoA C-acetyltransferase, acetoacetyl-CoA thiolase II
ACC	acetyl-CoA carboxylase
ACD	acyl-CoA dehydrogenase
ACS	acyl-CoA synthetase
AGC	aspartate/glutamate carrier antiporter or Aralar 1
αKG	□-ketoglutarate
ALAT	alanine aminotransferase
ALDP	adrenoleukodystrophy protein
AMPK	adenosine monophosphate activated protein kinase
ANLS	astrocyte-neuron lactate shuttle
ANT	adenine nucleotide transporter
ApN	aminopeptidase N
ApoE/D	apolipoprotein E/D
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCAA	branched chain amino acids
BCAT	branched chain aminotransferases
BDH	β-hydroxybutyrate dehydrogenase
CACT	carnitine/acylcarnitine translocase
cAMP	cyclic adenosine monophosphate
CAT	catalase

CIC	citrate isocitrate carrier
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CoA	coenzyme A
CPT	Carnitine palmitoyltransferase
CysGly	l-cysteine-l-glycine (CysGly)
EAAT	excitatory amino acid transporter
ECH	enoyl-CoA hydratase
ETC	electron transport chain
ETF	electron transferring flavoprotein
F2,6BP	fructose-2,6-bisphosphate
FA	fatty acid
FADH2	flavin adenine dinucleotide
FAO	fatty acid oxidation
FAT/CD36	fatty acid translocase
FATP	fatty acid transport protein
GABA	γ-aminobutyric acid
GABA-T	γ-aminobutyric acid transaminase
GAD	glutamate decarboxylase
GAT	γ-aminobutyric acid transporter
GC1	glutamate carrier or SLC25A22
GDH	glutamate dehydrogenase
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter or excitatory amino acid transporter 1
GLT-1	glutamate transporter 1 or excitatory amino acid transporter 2
GOT1	cytosolic aspartate aminotransferase
GOT2	mitochondrial aspartate aminotransferase
GS	glutamine synthetase

GSH	glutathione
HAD	3-hydroxyacyl-Coenzyme A dehydrogenase
HMCS1	β-hydroxy-β-methyl glutaryl (HMG)-CoA synthase
HMGCL	HMG-CoA lyase
IP_3	inositol triphosphate,
LCFA	long chain fatty acid
MCFA	medium chain fatty acids
MCT	monocarboxylate transporter
MPC	mitochondrial pyruvate carrier
Na^+/K^+ ATPase	sodium-potassium adenosine triphosphatase
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NDUFS1	nicotinamide adenine dinucleotide ubiquinone oxidoreductase core subunit S1
OXPHOS	oxidative phosphorylation
PAG	phosphate-activated glutaminase
PC	Pyruvate carboxylase
PDH	pyruvate dehydrogenase
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PFK1	phosphofructokinase 1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3
PhyH/Pahx	phytanoyl-CoA hydroxylase
PPAR	peroxisome proliferation activated receptor
PPP	pentose phosphate pathway
PUFA	poly-unsaturated fatty acid
Redox	reduction-oxidation
RET	reverse electron transfer
ROS	reactive oxygen specie

SCFA	short chain fatty acids
SCOT	succinyl-CoA-3-oxoacid-CoA transferase
SNAT	sodium-coupled neutral amino acid transporter
SSA	succinate semialdehyde
SSADH	succinate semialdehyde dehydrogenase
TCA cycle	tricarboxylic acid or Krebs cycle
TSP	transsulfuration pathway
VDAC	voltage-dependent anion channel
VLCFA	very long chain fatty acid
XAG-/EAAT3	aspartate-glutamate transporter
βHB	β-hydroxybutyrate
γGT	γ-glutamyl transpeptidase
Xc-/xCT	cystine-glutamate exchanger

3.9 Acknowledgements

This work was supported by the National Institutes of Health Grant P20RR17675 Centers of Biomedical Research Excellence (COBRE), the Research Council and the Office of Research at the University of Nebraska-Lincoln, and NSF Grant DBI-1461240.

3.10 References

Achanta, L.B., and Rae, C.D. (2017). beta-Hydroxybutyrate in the Brain: One Molecule, Multiple Mechanisms. *Neurochem Res* 42**,** 35-49.

Adina-Zada, A., Zeczycki, T.N., and Attwood, P.V. (2012). Regulation of the structure and activity of pyruvate carboxylase by acetyl CoA. *Arch Biochem Biophys* 519**,** 118-130.

Almeida, A., Moncada, S., and Bolanos, J.P. (2004). Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. *Nat Cell Biol* 6**,** 45-51.

Amoedo, N.D., Punzi, G., Obre, E., Lacombe, D., De Grassi, A., Pierri, C.L., and Rossignol, R. (2016). AGC1/2, the mitochondrial aspartate-glutamate carriers. *Biochim Biophys Acta* 1863**,** 2394-2412.

Anderson, C.M., and Stahl, A. (2013). SLC27 fatty acid transport proteins. *Mol Aspects Med* 34**,** 516-528.

Anderson, C.M., and Swanson, R.A. (2000). Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia* 32**,** 1-14.

Antonenkov, V.D., Croes, K., Waelkens, E., Van Veldhoven, P.P., and Mannaerts, G.P. (2000). Identification, purification and characterization of an acetoacetyl-CoA thiolase from rat liver peroxisomes. *Eur J Biochem* 267**,** 2981-2990.

Antonenkov, V.D., and Hiltunen, J.K. (2012). Transfer of metabolites across the peroxisomal membrane. *Biochim Biophys Acta* 1822**,** 1374-1386.

Aoyama, K., Watabe, M., and Nakaki, T. (2008). Regulation of neuronal glutathione synthesis. *J Pharmacol Sci* 108**,** 227-238.

Attwell, D., and Laughlin, S.B. (2001). An energy budget for signaling in the grey matter of the brain. *J Cereb Blood Flow Metab* 21**,** 1133-1145.

Auestad, N., Korsak, R.A., Morrow, J.W., and Edmond, J. (1991). Fatty acid oxidation and ketogenesis by astrocytes in primary culture. *J Neurochem* 56**,** 1376-1386.

Bak, L.K., Walls, A.B., Schousboe, A., and Waagepetersen, H.S. (2018). Astrocytic glycogen metabolism in the healthy and diseased brain. *J Biol Chem* 293**,** 7108-7116.

Basu-Modak, S., Braissant, O., Escher, P., Desvergne, B., Honegger, P., and Wahli, W. (1999). Peroxisome proliferator-activated receptor beta regulates acyl-CoA synthetase 2 in reaggregated rat brain cell cultures. *J Biol Chem* 274**,** 35881-35888.

Bauer, D.E., Jackson, J.G., Genda, E.N., Montoya, M.M., Yudkoff, M., and Robinson, M.B. (2012). The glutamate transporter, GLAST, participates in a macromolecular complex that supports glutamate metabolism. *Neurochem Int* 61**,** 566-574.

Bedner, P., Niessen, H., Odermatt, B., Kretz, M., Willecke, K., and Harz, H. (2006). Selective permeability of different connexin channels to the second messenger cyclic AMP. *J Biol Chem* 281**,** 6673-6681.

Belanger, M., Allaman, I., and Magistretti, P.J. (2011). Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. *Cell Metab* 14**,** 724-738.

Ben Haim, L., Carrillo-De Sauvage, M.A., Ceyzeriat, K., and Escartin, C. (2015). Elusive roles for reactive astrocytes in neurodegenerative diseases. *Front Cell Neurosci* 9**,** 278.

Ben Haim, L., and Rowitch, D.H. (2017). Functional diversity of astrocytes in neural circuit regulation. *Nat Rev Neurosci* 18**,** 31-41.

Bird, M.I., Munday, L.A., Saggerson, E.D., and Clark, J.B. (1985). Carnitine acyltransferase activities in rat brain mitochondria. Bimodal distribution, kinetic constants, regulation by malonyl-CoA and developmental pattern. *Biochem J* 226**,** 323-330.

Blazquez, C., Sanchez, C., Velasco, G., and Guzman, M. (1998). Role of carnitine palmitoyltransferase I in the control of ketogenesis in primary cultures of rat astrocytes. *J Neurochem* 71**,** 1597-1606.

Blazquez, C., Woods, A., De Ceballos, M.L., Carling, D., and Guzman, M. (1999). The AMP-activated protein kinase is involved in the regulation of ketone body production by astrocytes. *J Neurochem* 73**,** 1674-1682.

Bolanos, J.P. (2016). Bioenergetics and redox adaptations of astrocytes to neuronal activity. *J Neurochem* 139 Suppl 2**,** 115-125.

Booth, R.F., and Clark, J.B. (1981). Energy metabolism in rat brain: inhibition of pyruvate decarboxylation by 3-hydroxybutyrate in neonatal mitochondria. *J Neurochem* 37**,** 179-185.

Boumezbeur, F., Petersen, K.F., Cline, G.W., Mason, G.F., Behar, K.L., Shulman, G.I., and Rothman, D.L. (2010). The contribution of blood lactate to brain energy metabolism in humans measured by dynamic 13C nuclear magnetic resonance spectroscopy. *J Neurosci* 30**,** 13983-13991.

Bouzier-Sore, A.K., Voisin, P., Bouchaud, V., Bezancon, E., Franconi, J.M., and Pellerin, L. (2006). Competition between glucose and lactate as oxidative energy substrates in both neurons and astrocytes: a comparative NMR study. *Eur J Neurosci* 24**,** 1687-1694.

Calabrese, V., Ravagna, A., Colombrita, C., Scapagnini, G., Guagliano, E., Calvani, M., Butterfield, D.A., and Giuffrida Stella, A.M. (2005). Acetylcarnitine induces heme oxygenase in rat astrocytes and protects against oxidative stress: involvement of the transcription factor Nrf2. *J Neurosci Res* 79**,** 509-521.

Chechik, T., Roeder, L.M., Tildon, J.T., and Poduslo, S.E. (1987). Ketone body enzyme activities in purified neurons, astrocytes and oligodendroglia. *Neurochem Int* 10**,** 95-99.

Chen, C.T., Trepanier, M.O., Hopperton, K.E., Domenichiello, A.F., Masoodi, M., and Bazinet, R.P. (2014). Inhibiting mitochondrial beta-oxidation selectively reduces levels of nonenzymatic oxidative polyunsaturated fatty acid metabolites in the brain. *J Cereb Blood Flow Metab* 34**,** 376-379.

Chen, Y., Vartiainen, N.E., Ying, W., Chan, P.H., Koistinaho, J., and Swanson, R.A. (2001). Astrocytes protect neurons from nitric oxide toxicity by a glutathione-dependent mechanism. *J Neurochem* 77**,** 1601-1610.

Choi, I.Y., Tkac, I., Ugurbil, K., and Gruetter, R. (1999). Noninvasive measurements of [1-(13)C]glycogen concentrations and metabolism in rat brain in vivo. *J Neurochem* 73**,** 1300-1308.

Chowdhury, G.M., Jiang, L., Rothman, D.L., and Behar, K.L. (2014). The contribution of ketone bodies to basal and activity-dependent neuronal oxidation in vivo. *J Cereb Blood Flow Metab* 34**,** 1233-1242.

Chung, W.S., Allen, N.J., and Eroglu, C. (2015). Astrocytes Control Synapse Formation, Function, and Elimination. *Cold Spring Harb Perspect Biol* 7**,** a020370.

Cocco, T., Di Paola, M., Papa, S., and Lorusso, M. (1999). Arachidonic acid interaction with the mitochondrial electron transport chain promotes reactive oxygen species generation. *Free Radic Biol Med* 27**,** 51-59.

Courchesne-Loyer, A., Croteau, E., Castellano, C.A., St-Pierre, V., Hennebelle, M., and Cunnane, S.C. (2017). Inverse relationship between brain glucose and ketone metabolism in adults during short-term moderate dietary ketosis: A dual tracer quantitative positron emission tomography study. *J Cereb Blood Flow Metab* 37**,** 2485-2493.

Cullingford, T.E., Dolphin, C.T., Bhakoo, K.K., Peuchen, S., Canevari, L., and Clark, J.B. (1998). Molecular cloning of rat mitochondrial 3-hydroxy-3-methylglutaryl-CoA lyase and detection of the corresponding mRNA and of those encoding the remaining enzymes

comprising the ketogenic 3-hydroxy-3-methylglutaryl-CoA cycle in central nervous system of suckling rat. *Biochem J* 329 (Pt 2)**,** 373-381.

Cullingford, T.E., Dolphin, C.T., and Sato, H. (2002). The peroxisome proliferator-activated receptor alpha-selective activator ciprofibrate upregulates expression of genes encoding fatty acid oxidation and ketogenesis enzymes in rat brain. *Neuropharmacology* 42**,** 724-730.

Delgado-Esteban, M., Almeida, A., and Bolanos, J.P. (2000). D-Glucose prevents glutathione oxidation and mitochondrial damage after glutamate receptor stimulation in rat cortical primary neurons. *J Neurochem* 75**,** 1618-1624.

Diaz-Garcia, C.M., Mongeon, R., Lahmann, C., Koveal, D., Zucker, H., and Yellen, G. (2017). Neuronal Stimulation Triggers Neuronal Glycolysis and Not Lactate Uptake. *Cell Metab* 26**,** 361-374 e364.

Dringen, R., Gutterer, J.M., Gros, C., and Hirrlinger, J. (2001). Aminopeptidase N mediates the utilization of the GSH precursor CysGly by cultured neurons. *J Neurosci Res* 66**,** 1003-1008.

Dringen, R., Kranich, O., and Hamprecht, B. (1997). The gamma-glutamyl transpeptidase inhibitor acivicin preserves glutathione released by astroglial cells in culture. *Neurochem Res* 22**,** 727-733.

Dringen, R., Pfeiffer, B., and Hamprecht, B. (1999). Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. *J Neurosci* 19**,** 562-569.

Ebert, D., Haller, R.G., and Walton, M.E. (2003). Energy contribution of octanoate to intact rat brain metabolism measured by 13C nuclear magnetic resonance spectroscopy. *J Neurosci* 23**,** 5928-5935.

Edmond, J., Robbins, R.A., Bergstrom, J.D., Cole, R.A., and De Vellis, J. (1987). Capacity for substrate utilization in oxidative metabolism by neurons, astrocytes, and oligodendrocytes from developing brain in primary culture. *J Neurosci Res* 18**,** 551-561.

Escartin, C., Pierre, K., Colin, A., Brouillet, E., Delzescaux, T., Guillermier, M., Dhenain, M., Deglon, N., Hantraye, P., Pellerin, L., and Bonvento, G. (2007). Activation of astrocytes by CNTF induces metabolic plasticity and increases resistance to metabolic insults. *J Neurosci* 27**,** 7094-7104.

Farmer, W.T., Abrahamsson, T., Chierzi, S., Lui, C., Zaelzer, C., Jones, E.V., Bally, B.P., Chen, G.G., Theroux, J.F., Peng, J., Bourque, C.W., Charron, F., Ernst, C., Sjostrom, P.J., and Murai, K.K. (2016). Neurons diversify astrocytes in the adult brain through sonic hedgehog signaling. *Science* 351**,** 849-854.

Foster, D.W. (2012). Malonyl-CoA: the regulator of fatty acid synthesis and oxidation. *J Clin Invest* 122**,** 1958-1959.

Garber, A.J., and Ballard, F.J. (1969). Phosphoenolpyruvate synthesis and release by mitochondria from guinea pig liver. *J Biol Chem* 244**,** 4696-4703.

Garcia-Marin, V., Garcia-Lopez, P., and Freire, M. (2007). Cajal's contributions to glia research. *Trends Neurosci* 30**,** 479-487.

Genda, E.N., Jackson, J.G., Sheldon, A.L., Locke, S.F., Greco, T.M., O'donnell, J.C., Spruce, L.A., Xiao, R., Guo, W., Putt, M., Seeholzer, S., Ischiropoulos, H., and Robinson, M.B. (2011). Co-compartmentalization of the astroglial glutamate transporter, GLT-1, with glycolytic enzymes and mitochondria. *J Neurosci* 31**,** 18275-18288.

Goldberg, G.S., Moreno, A.P., and Lampe, P.D. (2002). Gap junctions between cells expressing connexin 43 or 32 show inverse permselectivity to adenosine and ATP. *J Biol Chem* 277**,** 36725-36730.

Goubert, E., Mircheva, Y., Lasorsa, F.M., Melon, C., Profilo, E., Sutera, J., Becq, H., Palmieri, F., Palmieri, L., Aniksztejn, L., and Molinari, F. (2017). Inhibition of the Mitochondrial Glutamate Carrier SLC25A22 in Astrocytes Leads to Intracellular Glutamate Accumulation. *Front Cell Neurosci* 11**,** 149.

Guzman, M., and Blazquez, C. (2001). Is there an astrocyte-neuron ketone body shuttle? *Trends Endocrinol Metab* 12**,** 169-173.

Halestrap, A.P. (1978). Pyruvate and ketone-body transport across the mitochondrial membrane. Exchange properties, pH-dependence and mechanism of the carrier. *Biochem J* 172**,** 377-387.

Halestrap, A.P., and Price, N.T. (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J* 343 Pt 2**,** 281-299.

Halim, N.D., Mcfate, T., Mohyeldin, A., Okagaki, P., Korotchkina, L.G., Patel, M.S., Jeoung, N.H., Harris, R.A., Schell, M.J., and Verma, A. (2010). Phosphorylation status of pyruvate dehydrogenase distinguishes metabolic phenotypes of cultured rat brain astrocytes and neurons. *Glia* 58**,** 1168-1176.

Hamilton, J.A., and Brunaldi, K. (2007). A model for fatty acid transport into the brain. *J Mol Neurosci* 33**,** 12-17.

Hein, S., Schonfeld, P., Kahlert, S., and Reiser, G. (2008). Toxic effects of X-linked adrenoleukodystrophy-associated, very long chain fatty acids on glial cells and neurons from rat hippocampus in culture. *Hum Mol Genet* 17**,** 1750-1761.

Herrero-Mendez, A., Almeida, A., Fernandez, E., Maestre, C., Moncada, S., and Bolanos, J.P. (2009). The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nat Cell Biol* 11**,** 747-752.

Hertz, L. (2011). Brain glutamine synthesis requires neuronal aspartate: a commentary. *J Cereb Blood Flow Metab* 31**,** 384-387.

Hirrlinger, J., and Dringen, R. (2005). Multidrug resistance protein 1-mediated export of glutathione and glutathione disulfide from brain astrocytes. *Methods Enzymol* 400**,** 395-409.

Hirrlinger, J., Schulz, J.B., and Dringen, R. (2002). Glutathione release from cultured brain cells: multidrug resistance protein 1 mediates the release of GSH from rat astroglial cells. *J Neurosci Res* 69**,** 318-326.

Ho, J.K., Moser, H., Kishimoto, Y., and Hamilton, J.A. (1995). Interactions of a very long chain fatty acid with model membranes and serum albumin. Implications for the pathogenesis of adrenoleukodystrophy. *J Clin Invest* 96**,** 1455-1463.

Hofmann, K., Rodriguez-Rodriguez, R., Gaebler, A., Casals, N., Scheller, A., and Kuerschner, L. (2017). Astrocytes and oligodendrocytes in grey and white matter regions of the brain metabolize fatty acids. *Sci Rep* 7**,** 10779.

Houten, S.M., and Wanders, R.J. (2010). A general introduction to the biochemistry of mitochondrial fatty acid beta-oxidation. *J Inherit Metab Dis* 33**,** 469-477.

Hovik, R., Brodal, B., Bartlett, K., and Osmundsen, H. (1991). Metabolism of acetyl-CoA by isolated peroxisomal fractions: formation of acetate and acetoacetyl-CoA. *J Lipid Res* 32**,** 993-999.

Hyder, F., Rothman, D.L., and Bennett, M.R. (2013). Cortical energy demands of signaling and nonsignaling components in brain are conserved across mammalian species and activity levels. *Proc Natl Acad Sci U S A* 110**,** 3549-3554.

Jackson, J.G., O'donnell, J.C., Takano, H., Coulter, D.A., and Robinson, M.B. (2014). Neuronal activity and glutamate uptake decrease mitochondrial mobility in astrocytes and position mitochondria near glutamate transporters. *J Neurosci* 34**,** 1613-1624.

Jackson, J.G., and Robinson, M.B. (2018). Regulation of mitochondrial dynamics in astrocytes: Mechanisms, consequences, and unknowns. *Glia* 66**,** 1213-1234.

Jakoby, P., Schmidt, E., Ruminot, I., Gutierrez, R., Barros, L.F., and Deitmer, J.W. (2014). Higher transport and metabolism of glucose in astrocytes compared with neurons: a multiphoton study of hippocampal and cerebellar tissue slices. *Cereb Cortex* 24**,** 222-231.

Jansen, G.A., and Wanders, R.J. (2006). Alpha-oxidation. *Biochim Biophys Acta* 1763**,** 1403-1412.

Jernberg, J.N., Bowman, C.E., Wolfgang, M.J., and Scafidi, S. (2017). Developmental regulation and localization of carnitine palmitoyltransferases (CPTs) in rat brain. *J Neurochem* 142**,** 407-419.

Jones, L.L., Mcdonald, D.A., and Borum, P.R. (2010). Acylcarnitines: role in brain. *Prog Lipid Res* 49**,** 61-75.

Kimura, S., and Amemiya, F. (1990). Brain and liver pathology in a patient with carnitine deficiency. *Brain Dev* 12**,** 436-439.

Komen, J.C., Distelmaier, F., Koopman, W.J., Wanders, R.J., Smeitink, J., and Willems, P.H. (2007). Phytanic acid impairs mitochondrial respiration through protonophoric action. *Cell Mol Life Sci* 64**,** 3271-3281.

Korshunov, S.S., Korkina, O.V., Ruuge, E.K., Skulachev, V.P., and Starkov, A.A. (1998). Fatty acids as natural uncouplers preventing generation of O2.- and H2O2 by mitochondria in the resting state. *FEBS Lett* 435**,** 215-218.

Koulakoff, A., Ezan, P., and Giaume, C. (2008). Neurons control the expression of connexin 30 and connexin 43 in mouse cortical astrocytes. *Glia* 56**,** 1299-1311.

Kranich, O., Hamprecht, B., and Dringen, R. (1996). Different preferences in the utilization of amino acids for glutathione synthesis in cultured neurons and astroglial cells derived from rat brain. *Neurosci Lett* 219**,** 211-214.

Krencik, R., Weick, J.P., Liu, Y., Zhang, Z.J., and Zhang, S.C. (2011). Specification of transplantable astroglial subtypes from human pluripotent stem cells. *Nat Biotechnol* 29**,** 528-534.

Kruska, N., Schonfeld, P., Pujol, A., and Reiser, G. (2015). Astrocytes and mitochondria from adrenoleukodystrophy protein (ABCD1)-deficient mice reveal that the adrenoleukodystrophy-associated very long-chain fatty acids target several cellular energy-dependent functions. *Biochim Biophys Acta* 1852**,** 925-936.

Kunnecke, B., Cerdan, S., and Seelig, J. (1993). Cerebral metabolism of [1,2-13C2]glucose and [U-13C4]3-hydroxybutyrate in rat brain as detected by 13C NMR spectroscopy. *NMR Biomed* 6**,** 264-277.

Lapato, A.S., and Tiwari-Woodruff, S.K. (2018). Connexins and pannexins: At the junction of neuro-glial homeostasis & disease. *J Neurosci Res* 96**,** 31-44.

Lavrentyev, E.N., Matta, S.G., and Cook, G.A. (2004). Expression of three carnitine palmitoyltransferase-I isoforms in 10 regions of the rat brain during feeding, fasting, and diabetes. *Biochem Biophys Res Commun* 315**,** 174-178.

Lee, J., and Wolfgang, M.J. (2012). Metabolomic profiling reveals a role for CPT1c in neuronal oxidative metabolism. *BMC Biochem* 13**,** 23.

Leke, R., and Schousboe, A. (2016). The Glutamine Transporters and Their Role in the Glutamate/GABA-Glutamine Cycle. *Adv Neurobiol* 13**,** 223-257.

Liu, L., Mackenzie, K.R., Putluri, N., Maletic-Savatic, M., and Bellen, H.J. (2017). The Glia-Neuron Lactate Shuttle and Elevated ROS Promote Lipid Synthesis in Neurons and Lipid Droplet Accumulation in Glia via APOE/D. *Cell Metab* 26**,** 719-737 e716.

Liu, L., Zhang, K., Sandoval, H., Yamamoto, S., Jaiswal, M., Sanz, E., Li, Z., Hui, J., Graham, B.H., Quintana, A., and Bellen, H.J. (2015). Glial lipid droplets and ROS induced by mitochondrial defects promote neurodegeneration. *Cell* 160**,** 177-190.

Lopes-Cardozo, M., Larsson, O.M., and Schousboe, A. (1986). Acetoacetate and glucose as lipid precursors and energy substrates in primary cultures of astrocytes and neurons from mouse cerebral cortex. *J Neurochem* 46**,** 773-778.

Lopez-Fabuel, I., Le Douce, J., Logan, A., James, A.M., Bonvento, G., Murphy, M.P., Almeida, A., and Bolanos, J.P. (2016). Complex I assembly into supercomplexes determines differential mitochondrial ROS production in neurons and astrocytes. *Proc Natl Acad Sci U S A* 113**,** 13063-13068.

Lovatt, D., Sonnewald, U., Waagepetersen, H.S., Schousboe, A., He, W., Lin, J.H., Han, X., Takano, T., Wang, S., Sim, F.J., Goldman, S.A., and Nedergaard, M. (2007). The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. *J Neurosci* 27**,** 12255-12266.

Lundgaard, I., Li, B., Xie, L., Kang, H., Sanggaard, S., Haswell, J.D., Sun, W., Goldman, S., Blekot, S., Nielsen, M., Takano, T., Deane, R., and Nedergaard, M. (2015). Direct neuronal glucose uptake heralds activity-dependent increases in cerebral metabolism. *Nat Commun* 6**,** 6807.

Machler, P., Wyss, M.T., Elsayed, M., Stobart, J., Gutierrez, R., Von Faber-Castell, A., Kaelin, V., Zuend, M., San Martin, A., Romero-Gomez, I., Baeza-Lehnert, F., Lengacher, S., Schneider, B.L., Aebischer, P., Magistretti, P.J., Barros, L.F., and Weber, B. (2016). In Vivo Evidence for a Lactate Gradient from Astrocytes to Neurons. *Cell Metab* 23**,** 94-102.

Macvicar, B.A., and Newman, E.A. (2015). Astrocyte regulation of blood flow in the brain. *Cold Spring Harb Perspect Biol* 7.

Magistretti, P.J., and Allaman, I. (2015). A cellular perspective on brain energy metabolism and functional imaging. *Neuron* 86**,** 883-901.

Magistretti, P.J., Pellerin, L., Rothman, D.L., and Shulman, R.G. (1999). Energy on demand. *Science* 283**,** 496-497.

Marszalek, J.R., Kitidis, C., Dararutana, A., and Lodish, H.F. (2004). Acyl-CoA synthetase 2 overexpression enhances fatty acid internalization and neurite outgrowth. *J Biol Chem* 279**,** 23882-23891.

Mashek, D.G., Li, L.O., and Coleman, R.A. (2007). Long-chain acyl-CoA synthetases and fatty acid channeling. *Future Lipidol* 2**,** 465-476.

Matthews, G.D., Gur, N., Koopman, W.J., Pines, O., and Vardimon, L. (2010). Weak mitochondrial targeting sequence determines tissue-specific subcellular localization of glutamine synthetase in liver and brain cells. *J Cell Sci* 123**,** 351-359.

Mckenna, M.C., Sonnewald, U., Huang, X., Stevenson, J., and Zielke, H.R. (1996). Exogenous glutamate concentration regulates the metabolic fate of glutamate in astrocytes. *J Neurochem* 66**,** 386-393.

Mckenna, M.C., Stridh, M.H., Mcnair, L.F., Sonnewald, U., Waagepetersen, H.S., and Schousboe, A. (2016). Glutamate oxidation in astrocytes: Roles of glutamate dehydrogenase and aminotransferases. *J Neurosci Res* 94**,** 1561-1571.

Morris, A.A. (2005). Cerebral ketone body metabolism. *J Inherit Metab Dis* 28**,** 109-121.

Murin, R., Cesar, M., Kowtharapu, B.S., Verleysdonk, S., and Hamprecht, B. (2009). Expression of pyruvate carboxylase in cultured oligodendroglial, microglial and ependymal cells. *Neurochem Res* 34**,** 480-489.

Niessen, H., Harz, H., Bedner, P., Kramer, K., and Willecke, K. (2000). Selective permeability of different connexin channels to the second messenger inositol 1,4,5-trisphosphate. *J Cell Sci* 113 (Pt 8)**,** 1365-1372.

Nissen, J.D., Lykke, K., Bryk, J., Stridh, M.H., Zaganas, I., Skytt, D.M., Schousboe, A., Bak, L.K., Enard, W., Paabo, S., and Waagepetersen, H.S. (2017). Expression of the human isoform of glutamate dehydrogenase, hGDH2, augments TCA cycle capacity and oxidative metabolism of glutamate during glucose deprivation in astrocytes. *Glia* 65**,** 474-488.

Nissen, J.D., Pajecka, K., Stridh, M.H., Skytt, D.M., and Waagepetersen, H.S. (2015). Dysfunctional TCA-Cycle Metabolism in Glutamate Dehydrogenase Deficient Astrocytes. *Glia* 63**,** 2313-2326.

Norenberg, M.D., and Martinez-Hernandez, A. (1979). Fine structural localization of glutamine synthetase in astrocytes of rat brain. *Brain Res* 161**,** 303-310.

Obici, S., Feng, Z., Arduini, A., Conti, R., and Rossetti, L. (2003). Inhibition of hypothalamic carnitine palmitoyltransferase-1 decreases food intake and glucose production. *Nat Med* 9**,** 756-761.

Olivier, L.M., Kovacs, W., Masuda, K., Keller, G.A., and Krisans, S.K. (2000). Identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes. AA-CoA thiolase, hmg-coa synthase, MPPD, and FPP synthase. *J Lipid Res* 41**,** 1921-1935.

Olsen, G.M., and Sonnewald, U. (2015). Glutamate: Where does it come from and where does it go? *Neurochem Int* 88**,** 47-52.

Orthmann-Murphy, J.L., Abrams, C.K., and Scherer, S.S. (2008). Gap junctions couple astrocytes and oligodendrocytes. *J Mol Neurosci* 35**,** 101-116.

Owen, O.E., Kalhan, S.C., and Hanson, R.W. (2002). The key role of anaplerosis and cataplerosis for citric acid cycle function. *J Biol Chem* 277**,** 30409-30412.

Owen, O.E., Morgan, A.P., Kemp, H.G., Sullivan, J.M., Herrera, M.G., and Cahill, G.F., Jr. (1967). Brain metabolism during fasting. *J Clin Invest* 46**,** 1589-1595.

Pajecka, K., Nissen, J.D., Stridh, M.H., Skytt, D.M., Schousboe, A., and Waagepetersen, H.S. (2015). Glucose replaces glutamate as energy substrate to fuel glutamate uptake in glutamate dehydrogenase-deficient astrocytes. *J Neurosci Res* 93**,** 1093-1100.

Panov, A., Orynbayeva, Z., Vavilin, V., and Lyakhovich, V. (2014). Fatty acids in energy metabolism of the central nervous system. *Biomed Res Int* 2014**,** 472459.

Paradies, G., and Papa, S. (1977). On the kinetics and substrate specificity of the pyruvate translocator in rat liver mitochondria. *Biochim Biophys Acta* 462**,** 333-346.

Patel, A.B., Lai, J.C., Chowdhury, G.M., Hyder, F., Rothman, D.L., Shulman, R.G., and Behar, K.L. (2014). Direct evidence for activity-dependent glucose phosphorylation in neurons with implications for the astrocyte-to-neuron lactate shuttle. *Proc Natl Acad Sci U S A* 111**,** 5385-5390.

Pellerin, L., and Magistretti, P.J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A* 91**,** 10625-10629.

Riveros, N., Fiedler, J., Lagos, N., Munoz, C., and Orrego, F. (1986). Glutamate in rat brain cortex synaptic vesicles: influence of the vesicle isolation procedure. *Brain Res* 386**,** 405-408.

Rolfe, D.F., and Brown, G.C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol Rev* 77**,** 731-758.

Romano, A., Koczwara, J.B., Gallelli, C.A., Vergara, D., Micioni Di Bonaventura, M.V., Gaetani, S., and Giudetti, A.M. (2017). Fats for thoughts: An update on brain fatty acid metabolism. *Int J Biochem Cell Biol* 84**,** 40-45.

Rose, E.M., Koo, J.C., Antflick, J.E., Ahmed, S.M., Angers, S., and Hampson, D.R. (2009). Glutamate transporter coupling to Na,K-ATPase. *J Neurosci* 29**,** 8143-8155.

Sagara, J.I., Miura, K., and Bannai, S. (1993). Maintenance of neuronal glutathione by glial cells. *J Neurochem* 61**,** 1672-1676.

Schmoll, D., Fuhrmann, E., Gebhardt, R., and Hamprecht, B. (1995). Significant amounts of glycogen are synthesized from 3-carbon compounds in astroglial primary cultures from mice with participation of the mitochondrial phosphoenolpyruvate carboxykinase isoenzyme. *Eur J Biochem* 227**,** 308-315.

Schonfeld, P., and Reiser, G. (2013). Why does brain metabolism not favor burning of fatty acids to provide energy? Reflections on disadvantages of the use of free fatty acids as fuel for brain. *J Cereb Blood Flow Metab* 33**,** 1493-1499.

Schonfeld, P., and Reiser, G. (2016). Brain Lipotoxicity of Phytanic Acid and Very Long-chain Fatty Acids. Harmful Cellular/Mitochondrial Activities in Refsum Disease and X-Linked Adrenoleukodystrophy. *Aging Dis* 7**,** 136-149.

Schonfeld, P., and Reiser, G. (2017). Brain energy metabolism spurns fatty acids as fuel due to their inherent mitotoxicity and potential capacity to unleash neurodegeneration. *Neurochem Int* 109**,** 68-77.

Schonfeld, P., Wieckowski, M.R., Lebiedzinska, M., and Wojtczak, L. (2010). Mitochondrial fatty acid oxidation and oxidative stress: lack of reverse electron transfer-associated production of reactive oxygen species. *Biochim Biophys Acta* 1797**,** 929-938.

Schonfeld, P., and Wojtczak, L. (2007). Fatty acids decrease mitochondrial generation of reactive oxygen species at the reverse electron transport but increase it at the forward transport. *Biochim Biophys Acta* 1767**,** 1032-1040.

Schonfeld, P., and Wojtczak, L. (2008). Fatty acids as modulators of the cellular production of reactive oxygen species. *Free Radic Biol Med* 45**,** 231-241.

Schonfeld, P., and Wojtczak, L. (2016). Short- and medium-chain fatty acids in energy metabolism: the cellular perspective. *J Lipid Res* 57**,** 943-954.

Schousboe, A., Bak, L.K., and Waagepetersen, H.S. (2013). Astrocytic Control of Biosynthesis and Turnover of the Neurotransmitters Glutamate and GABA. *Front Endocrinol (Lausanne)* 4**,** 102.

Seib, T.M., Patel, S.A., and Bridges, R.J. (2011). Regulation of the system x(C)-cystine/glutamate exchanger by intracellular glutathione levels in rat astrocyte primary cultures. *Glia* 59**,** 1387-1401.

Seifert, E.L., Estey, C., Xuan, J.Y., and Harper, M.E. (2010). Electron transport chain-dependent and -independent mechanisms of mitochondrial H2O2 emission during long-chain fatty acid oxidation. *J Biol Chem* 285**,** 5748-5758.

Shank, R.P., Bennett, G.S., Freytag, S.O., and Campbell, G.L. (1985). Pyruvate carboxylase: an astrocyte-specific enzyme implicated in the replenishment of amino acid neurotransmitter pools. *Brain Res* 329**,** 364-367.

Shanker, G., Allen, J.W., Mutkus, L.A., and Aschner, M. (2001). The uptake of cysteine in cultured primary astrocytes and neurons. *Brain Res* 902**,** 156-163.

Shih, A.Y., Johnson, D.A., Wong, G., Kraft, A.D., Jiang, L., Erb, H., Johnson, J.A., and Murphy, T.H. (2003). Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. *J Neurosci* 23**,** 3394-3406.

Sibson, N.R., Dhankhar, A., Mason, G.F., Rothman, D.L., Behar, K.L., and Shulman, R.G. (1998). Stoichiometric coupling of brain glucose metabolism and glutamatergic neuronal activity. *Proc Natl Acad Sci U S A* 95**,** 316-321.

Sierra, A.Y., Gratacos, E., Carrasco, P., Clotet, J., Urena, J., Serra, D., Asins, G., Hegardt, F.G., and Casals, N. (2008). CPT1c is localized in endoplasmic reticulum of neurons and has carnitine palmitoyltransferase activity. *J Biol Chem* 283**,** 6878-6885.

Smith, D., Pernet, A., Hallett, W.A., Bingham, E., Marsden, P.K., and Amiel, S.A. (2003). Lactate: a preferred fuel for human brain metabolism in vivo. *J Cereb Blood Flow Metab* 23**,** 658-664.

Song, H., Stevens, C.F., and Gage, F.H. (2002). Astroglia induce neurogenesis from adult neural stem cells. *Nature* 417**,** 39-44.

Sonnewald, U., Westergaard, N., Petersen, S.B., Unsgard, G., and Schousboe, A. (1993). Metabolism of [U-13C]glutamate in astrocytes studied by 13C NMR spectroscopy: incorporation of more label into lactate than into glutamine demonstrates the importance of the tricarboxylic acid cycle. *J Neurochem* 61**,** 1179-1182.

Stark, R., and Kibbey, R.G. (2014). The mitochondrial isoform of phosphoenolpyruvate carboxykinase (PEPCK-M) and glucose homeostasis: has it been overlooked? *Biochim Biophys Acta* 1840**,** 1313-1330.

Stark, R., Pasquel, F., Turcu, A., Pongratz, R.L., Roden, M., Cline, G.W., Shulman, G.I., and Kibbey, R.G. (2009). Phosphoenolpyruvate cycling via mitochondrial phosphoenolpyruvate carboxykinase links anaplerosis and mitochondrial GTP with insulin secretion. *J Biol Chem* 284**,** 26578-26590.

Stephen, T.L., Higgs, N.F., Sheehan, D.F., Al Awabdh, S., Lopez-Domenech, G., Arancibia-Carcamo, I.L., and Kittler, J.T. (2015). Miro1 Regulates Activity-Driven Positioning of Mitochondria within Astrocytic Processes Apposed to Synapses to Regulate Intracellular Calcium Signaling. *J Neurosci* 35**,** 15996-16011.

Sugiura, A., Mattie, S., Prudent, J., and Mcbride, H.M. (2017). Newly born peroxisomes are a hybrid of mitochondrial and ER-derived pre-peroxisomes. *Nature* 542**,** 251-254.

Sun, X., Shih, A.Y., Johannssen, H.C., Erb, H., Li, P., and Murphy, T.H. (2006). Two-photon imaging of glutathione levels in intact brain indicates enhanced redox buffering in developing neurons and cells at the cerebrospinal fluid and blood-brain interface. *J Biol Chem* 281**,** 17420-17431.

Supplie, L.M., Duking, T., Campbell, G., Diaz, F., Moraes, C.T., Gotz, M., Hamprecht, B., Boretius, S., Mahad, D., and Nave, K.A. (2017). Respiration-Deficient Astrocytes Survive As Glycolytic Cells In Vivo. *J Neurosci* 37**,** 4231-4242.

Takahashi, S., Iizumi, T., Mashima, K., Abe, T., and Suzuki, N. (2014). Roles and regulation of ketogenesis in cultured astroglia and neurons under hypoxia and hypoglycemia. *ASN Neuro* 6.

Thevenet, J., De Marchi, U., Domingo, J.S., Christinat, N., Bultot, L., Lefebvre, G., Sakamoto, K., Descombes, P., Masoodi, M., and Wiederkehr, A. (2016). Medium-chain fatty acids inhibit mitochondrial metabolism in astrocytes promoting astrocyte-neuron lactate and ketone body shuttle systems. *FASEB J* 30**,** 1913-1926.

Troffer-Charlier, N., Doerflinger, N., Metzger, E., Fouquet, F., Mandel, J.L., and Aubourg, P. (1998). Mirror expression of adrenoleukodystrophy and adrenoleukodystrophy related genes in mouse tissues and human cell lines. *Eur J Cell Biol* 75**,** 254-264.

Trompier, D., Vejux, A., Zarrouk, A., Gondcaille, C., Geillon, F., Nury, T., Savary, S., and Lizard, G. (2014). Brain peroxisomes. *Biochimie* 98**,** 102-110.

Van Hall, G., Stromstad, M., Rasmussen, P., Jans, O., Zaar, M., Gam, C., Quistorff, B., Secher, N.H., and Nielsen, H.B. (2009). Blood lactate is an important energy source for the human brain. *J Cereb Blood Flow Metab* 29**,** 1121-1129.

Vasquez, O.L., Almeida, A., and Bolanos, J.P. (2001). Depletion of glutathione up-regulates mitochondrial complex I expression in glial cells. *J Neurochem* 76**,** 1593-1596.

Vaughn, A.E., and Deshmukh, M. (2008). Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c. *Nat Cell Biol* 10**,** 1477-1483.

Verkhratsky, A., Matteoli, M., Parpura, V., Mothet, J.P., and Zorec, R. (2016). Astrocytes as secretory cells of the central nervous system: idiosyncrasies of vesicular secretion. *EMBO J* 35**,** 239-257.

Vitvitsky, V., Thomas, M., Ghorpade, A., Gendelman, H.E., and Banerjee, R. (2006). A functional transsulfuration pathway in the brain links to glutathione homeostasis. *J Biol Chem* 281**,** 35785-35793.

Volboueva, L.A., Suh, S.W., Swanson, R.A., and Giffard, R.G. (2007). Inhibition of mitochondrial function in astrocytes: implications for neuroprotection. *J Neurochem* 102**,** 1383-1394.

Von Bartheld, C.S., Bahney, J., and Herculano-Houzel, S. (2016). The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting. *J Comp Neurol* 524**,** 3865-3895.

Von Bernhardi, R., Eugenin-Von Bernhardi, J., Flores, B., and Eugenin Leon, J. (2016). Glial Cells and Integrity of the Nervous System. *Adv Exp Med Biol* 949**,** 1-24.

Wang, X.F., and Cynader, M.S. (2000). Astrocytes provide cysteine to neurons by releasing glutathione. *J Neurochem* 74**,** 1434-1442.

Weightman Potter, P.G., Vlachaki Walker, J.M., Robb, J.L., Chilton, J.K., Williamson, R., Randall, A.D., Ellacott, K.L.J., and Beall, C. (2019). Basal fatty acid oxidation increases after recurrent low glucose in human primary astrocytes. *Diabetologia* 62**,** 187-198.

Wolfgang, M.J., Kurama, T., Dai, Y., Suwa, A., Asaumi, M., Matsumoto, S., Cha, S.H., Shimokawa, T., and Lane, M.D. (2006). The brain-specific carnitine palmitoyltransferase-1c regulates energy homeostasis. *Proc Natl Acad Sci U S A* 103**,** 7282-7287.

Wong-Riley, M.T. (1989). Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. *Trends Neurosci* 12**,** 94-101.

Yang, S.Y., He, X.Y., and Schulz, H. (1987). Fatty acid oxidation in rat brain is limited by the low activity of 3-ketoacyl-coenzyme A thiolase. *J Biol Chem* 262**,** 13027-13032.

Yang, Y., Gozen, O., Watkins, A., Lorenzini, I., Lepore, A., Gao, Y., Vidensky, S., Brennan, J., Poulsen, D., Won Park, J., Li Jeon, N., Robinson, M.B., and Rothstein, J.D. (2009). Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. *Neuron* 61**,** 880-894.

Yu, A.C., Drejer, J., Hertz, L., and Schousboe, A. (1983). Pyruvate carboxylase activity in primary cultures of astrocytes and neurons. *J Neurochem* 41**,** 1484-1487.

Zielke, H.R., Zielke, C.L., and Baab, P.J. (2009). Direct measurement of oxidative metabolism in the living brain by microdialysis: a review. *J Neurochem* 109 Suppl 1**,** 24-29.

CHAPTER 4

ASTROCYTES DEPEND UPON MITOCHONDRIA DURING ARSENIC EXPOSURE

4.1 Introduction

As discussed in chapters 2 and 3, astrocytes and neurons perform specialized metabolic functions for synaptic transmission, with astrocytes primarily maintaining homeostasis. Astrocyte processes envelope the capillary blood brain barrier (BBB) (Mathiisen et al., 2010), and become the first central nervous system (CNS) cells to encounter toxic compounds entering the brain. While numerous studies have documented the effects of xenobiotics on neuronal function, far fewer have delved into the effects of xenobiotics on astrocytes. While astrocytes have demonstrated resilience in the face of xenobiotic challenges compared to other CNS cell types (Hollensworth et al., 2000;Rathinam et al., 2012), how astrocyte metabolism changes to compensate for xenobiotic exposure has not been fully explored. In particular, the role of mitochondrial metabolism in astrocytes has often been overlooked, in part because of the aforementioned resilience of astrocytes to xenobiotic challenges and the demonstrated glycolytic nature of astrocytes (Supplie et al., 2017). Given the interconnected nature of astrocyte-neuron metabolism, it is imperative to understand what metabolic alterations are induced in astrocytes by xenobiotics to understand how xenobiotic toxicity affects neurotransmission.

Arsenic is a metalloid element with a widespread, variable distribution throughout the Earth's crust. The high aqueous solubility of inorganic arsenicals allows for high groundwater contamination (Nordstrom, 2002;Duker et al., 2005). One of the best documented exposure to arsenic contaminated water occurred in the 1980s in Bangladesh, when shallow wells were constructed to increase access to clean water. The issue went largely unaddressed until 1998, and water analysis revealed that 34.9% of the 19,845 wells of the flood plains had arsenic contamination above the World Health Organization's (WHO) maximum permissible limit of 50

µg/L, with the highest measured concentration being 4730 µg/L (Chakraborti et al., 2015). No level of arsenic contamination is without risk, as illustrated in a study of verbal, intellect quotient, and long term memory on Bangladeshi children found that the inverse correlation between each test and arsenic water contamination could be observed even between 0.1-5.5ug/L and 5.6-50ug/L groups (Wasserman et al., 2004). Further, arsenic associated skin lesions have been reported following chronic exposure to water containing less than 10ug/L of arsenic (Ahsan et al., 2000;Ahsan et al., 2006), however the link to skin cancer at these concentrations is still debated (see (Hong et al., 2014) for a review on chronic arsenic exposure symptoms). The arsenic contaminated water was also used in irrigation, and an ensuing analysis of regions with high arsenic contamination (200-500 µg/L) found a mean arsenic accumulation of 358 µg/kg and 333 µg/kg in rice and vegetables, respectively. Further, 87% of the arsenic in rice and 96% of the arsenic in vegetables were in inorganic forms (Smith et al., 2006). Arsenicals, such as p-arsanilic acid and nitarsone, that are not readily absorbed into animals are used in animal feed to mitigate protozoan infections and are excreted. However, when manure from these animals is used as fertilizer, the arsenicals are absorbed into agricultural products such as rice. The plants then convert the arsenicals into forms that are readily absorbed and toxic to animals, such as arsenite (As^{III}), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and arsenate (As^{V}) (Geng et al., 2017).

Inorganic arsenic typically exists in two oxidation states, the pentavalent arsenate (As^{V}) that predominates in oxygenated or oxidizing environments, and the trivalent arsenite (As^{III}) that predominates in reductive environments (Duker et al., 2005). Arsenite enters the cell through aquaglycoporins (Liu et al., 2004) and glucose transporters (Liu et al., 2006), while arsenate enters through phosphate transporters (Villa-Bellosta and Sorribas, 2010). Early studies on mammalian arsenic exposure revealed that DMA and MMA in the pentavalent form (DMA^{V} and MMA^{V}) were present in urine. DMA^{V} and MMA^{V} displayed reduced cytotoxicity, but were later

found to possess carcinogenic properties. It was later discovered that the trivalent forms of the metabolites (DMA^{III} and MMA^{III}) were also present in urine and were more carcinogenic than the pentavalent forms (reviewed in (Drobna et al., 2009)). Further, the principal enzyme in methylation of inorganic arsenic in humans, arsenic methyltransferase (AS3MT), uses trivalent arsenicals as a substrate. Further, methylation is promoted in the presence of GSH and reductases (Waters et al., 2004), reinforcing the proposed model whereby arsenicals alternate their oxidation state during methylation (Challenger, 1947). Further supporting this notion is the observed preference for glutathionylated arsenicals by AS3MT (Hayakawa et al., 2005), and the discovery of glutathionylated arsenicals in the urine of mice deficient in ɣ-glutamyltranspeptidase (ɣGT)(Kala et al., 2004).

In the brain, AS3MT has been shown to be highly expressed in astrocytes compared to neurons (Li et al., 2016), but it has also been reported that cultured astrocytes do not produce significant quantities of methylated arsenic species (Koehler et al., 2014). Nevertheless, the literature also indicates that methylated arsenic is produced in brain slices exposed to arsenic (Rodriguez et al., 2005), and that astrocytes are highly resistant to the effects of methylated arsenicals (Jin et al., 2004;Meyer et al., 2013). This methylation vs. glutathionylation distinction is important, as a methylation process with a glutathionylated arsenical intermediate would be expected to release GSH, or a derivative of GSH, back into the intra- or extracellular compartments. Further, triglutathionylated arsenical has been proposed to be exported by MRP1 (Leslie et al., 2004). However, this experiment was performed in HeLa cells and MRP1 has altered substrate specificity and kinetics depending on cell type (Banerjee et al., 2018), and corresponding post-translational modifications (Shukalek et al., 2016).

The importance of determining the mechanism of arsenic toxicity in the cells of the CNS is underscored by the neurodegenerative and cognitive impairment observed with chronic arsenic exposure. Multiple inverse correlations have been found between intelligence quotient (IQ)

parameters and environmental arsenic exposure during childhood (Rosado et al., 2007;Dong and Su, 2009;Hamadani et al., 2011;Wasserman et al., 2014). How this occurs has not been determined, though there is evidence that arsenic exposure alters neurotransmitter homeostasis (Yadav et al., 2010;Jiang et al., 2014;Ramos-Chavez et al., 2015), long term potentiation (Kruger et al., 2009), and mitochondrial function (Hu et al., 1998;Prakash et al., 2015). Arsenic has also been proposed to induce the production of reactive oxygen species (ROS) in the brain, particularly in neurons (Piao et al., 2005;Dwivedi and Flora, 2011;Flora, 2011;Prakash and Kumar, 2016), as well as alter the expression of excitatory amino acid transporters (Castro-Coronel et al., 2011;Zhao et al., 2012).

The effects of arsenic induced mitochondrial dysfunction have been difficult to elucidate. Isolated rat liver mitochondria have been shown to reduce arsenate to arsenite in a manner that is dependent upon electron transport chain activity (Nemeti and Gregus, 2002). Further, arsenite, and to a lesser extent methylated arsenite species, have been shown to inhibit both purified pyruvate dehydrogenase and □KG activity by binding to the reduced lipoic acid moieties (Bergquist et al., 2009). In line with this observation, arsenite exposure has been shown to reduce mitochondrial complex activities and oxygen consumption, while increasing the mutation rate of nuclear and mitochondrial DNA in a manner dependent upon mitochondrial activity (Liu et al., 2005;Partridge et al., 2007;Hosseini et al., 2013). The DNA damage does not appear to be a direct effect of arsenic on DNA repair enzymes (Hu et al., 1998). Instead, DNA damage may be indirect effect of the ROS generated from arsenic exposure (Ramanathan et al., 2003;Hosseini et al., 2013;Prakash et al., 2015). However, ROS may not be directly produced by arsenic, and it has been proposed that GSH depletion during arsenite exposure is the source of the observed ROS (Han et al., 2008).

In this chapter, arsenic was used as a representative xenobiotic to explore alterations to astrocyte metabolism during xenobiotic exposure. The experiments confirm previous reports that

astrocyte arsenic detoxification is glutathione (GSH) and multi-drug resistance associated protein 1 (MRP1) dependent, and that astrocytes remain viable when challenged with mitochondrial toxins. The experiments highlight that astrocyte viability is compromised during arsenic exposure by the loss of mitochondrial functions, inhibition of mitochondrial carbon inputs from pyruvate and fatty acids, and loss of aminotransferase activity. Further, arsenic exposure causes astrocytes to efflux glutamate to the media, generating extracellular glutamate concentrations that would be considered excitotoxic to neurons.

4.2 Materials and Methods

4.2.1 Cell Culture

Primary cortical astrocytes from post-natal day 1-2 C57Bl/6 mice were obtained from stocks previously frozen by Dr Annadurai Anandhan and Dr Carla Garza-Lombo, following the protocol of (Waagepetersen et al., 2002). Astrocytes were cultured in Dulbecco's Modified Eagle Medium with nutrient mixture F-12 (DMEM:f12, Hyclone) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), and 200 U/ml penicillin + 200 µg/ml streptomycin (P/S, Lonza). Cultured astrocytes were kept at 37°C in 5% carbon dioxide (CO_2) humidified incubator. Astrocytes were used up to the third passage.

4.2.2 Flow Cytometry

Astrocytes were seeded into 12-well plates at a density of 84,000 cells/ml. Fresh media supplemented with treatments was provided once the astrocytes reached 95% confluency. Cells were treated for 48hrs under the conditions above. Cell viability was determined with propidium iodide uptake (PI, 1 µg/ml) (Life Technologies) detecting loss of plasma membrane, and monochlorobimane (mBCl, 10 µM) (Molecular Probes) measuring intracellular GSH content. Flow cytometry was performed as described previously (Anandhan et al., 2017).

4.2.3 ICP-MS

Astrocytes were seeded into 12-well plates at a density of 84,000 cells/ml in DMEM:f12 supplemented medium as described above. Fresh DMEM:f12 supplemented with treatments was provided once the astrocytes reached 95% confluency. At the indicated time points, the media was removed and the cell trypsinized. Twice, the cells were pelleted, the supernatant removed, and the pellet gently resuspended in phosphate buffered saline (PBS). Half of each sample was then sent for ICP-MS, while the other half was used for normalization by a Pierce bicinchoninic acid (BCA) protein assay (Thermo). ICP-MS was performed as per (Anandhan et al., 2015).

4.2.4 Glutamate Quantification

Astrocytes were seeded into 12-well plates at a density of 84,000 cells/ml in DMEM:f12 supplemented medium as described above. Once the astrocytes reached 95% confluency, the cells were washed with prewarmed PBS and given Neurobasal A media supplemented with 1% B27, 2.5 mM glutamine and treatments for 24 hrs. The Neurobasal A media was removed and the sample was centrifuged at 300 g for 5 mins to pellet and remove any loose cells. Neurobasal media was then used in a Glutamate-Glo kit (Promega) as per manufacturer's instructions for 50 µl of samples. Media samples were diluted fourfold in PBS to keep measurements within the linear range of the kit. Results were normalized to total media volume at the time of collection, and to total protein content of the cells by a Pierce bicinchoninic acid (BCA) protein assay (Thermo).

4.2.5 ATP Quantification

Astrocytes were seeded into 96-well plates at a density of 7000 cells/well in DMEM:f12 supplemented medium as described above. Fresh DMEM:f12 supplemented with treatments was

provided once the astrocytes reached 95% confluency. After 24 hrs of treatment, Cell-Titer Assay (Promega) was performed as per manufacturer's instructions to quantify the total ATP content of each well.

4.2.6 Seahorse Mitochondrial Oxygen Consumption

Oxygen consumption (OCR, pmol/min) was determined using XFe24 Analyzer (Seahorse Biosciences) as per (Anandhan et al., 2017) with modifications outlined below. Astrocytes were grown to confluency in DMEM:f12 supplemented media as previously described and before additional media containing treatment factors was added. Treatment occurred in a CO_2-less incubator at 37°C for 24 hrs. After treatment, cells were washed and incubated for 1hr in HCO_3 and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) free DMEM (Gibco) supplemented with 2.5 mM glutamine, 17.5mM glucose, 1mM pyruvate and treatment factors prior to OCR measurements. OCR measurements were conducted using a final concentration of 1µM oligomycin, 1µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), and a mixture of 0.5 µM rotenone and 0.5 µM antimycin A. Measurements were normalized to total protein concentration. OCR was used as an indicator of mitochondrial function and capacity, as described by the manufacturer's protocols.

4.2.7 NMR Metabolomics

Astrocytes were seeded onto 100 mm dishes at a density of $1.2x10^6$ cells/9ml. Upon reaching 95% confluency, cells were washed twice with 1 ml prewarmed PBS. DMEM media supplemented with 17.5 mM U-^{13}C glucose, 1 mM pyruvate, and 2.5 mM glutamine in addition to 10% FBS and P/S, and sodium arsenite. For glutamate metabolomics, 17.5 mM unlabeled dextrose was used, and 100 µM U-^{13}C/^{15}N glutamate was added. Following 24 hrs, cells and

media were collected by Jade Woods and Alexandra Crook, with 2D ^{1}H-^{13}C NMR HSQC experiments were performed as per (Bhinderwala et al., 2019).

4.3 Results

4.3.1 Arsenic Exposure Creates a Reduced Intracellular State

Previous reports have implicated the glutathionylation of arsenicals in the detoxification process of many mammalian cell types, including astrocytes, that is either directly excreted or converted to a methylated arsenical for export (Cui et al., 2004;Leslie et al., 2004;Hayakawa et al., 2005). Flow cytometry was used to measure intracellular GSH and membrane integrity of astrocytes with various arsenic concentrations. In this regards, the results were used to determine if the loss of GSH was a measurable characteristic of arsenic toxicity by flow cytometry. Viable cells were defined as cells that are mBCl positive and PI negative. Preliminary data from Anandhan Annadurai showed that GSH depletion proceeded loss of membrane integrity (**Figure 4.1A**). In line with previous reports, low concentrations of arsenic increased the intracellular GSH concentration compared to the non-treated controls (**Figure 4.1B**).

4.3.2 Arsenic Exposure Increases Glutamate Synthesis from Glucose

Nuclear magnetic resonance (NMR) was used to create a metabolomic profile of astrocytes cultured with U-^{13}C-glucose at the previously utilized arsenic concentrations by Anandhan Annadurai and Shulei Lei. As shown in Figure 4.2A, the intracellular compartment demonstrated a significant fold increase in 3-phosphoglycerate (a precursor to both glycine and cysteine synthesis) during arsenic exposure. Conversely, the concentration of fructose-6-phosphate first decreased at low arsenic concentrations and then increased at higher arsenic concentrations. Notably, intracellular lactate remained initially constant and only decreased at the highest arsenic concentrations, while UDP-glucose decreased at all tested arsenic concentrations.

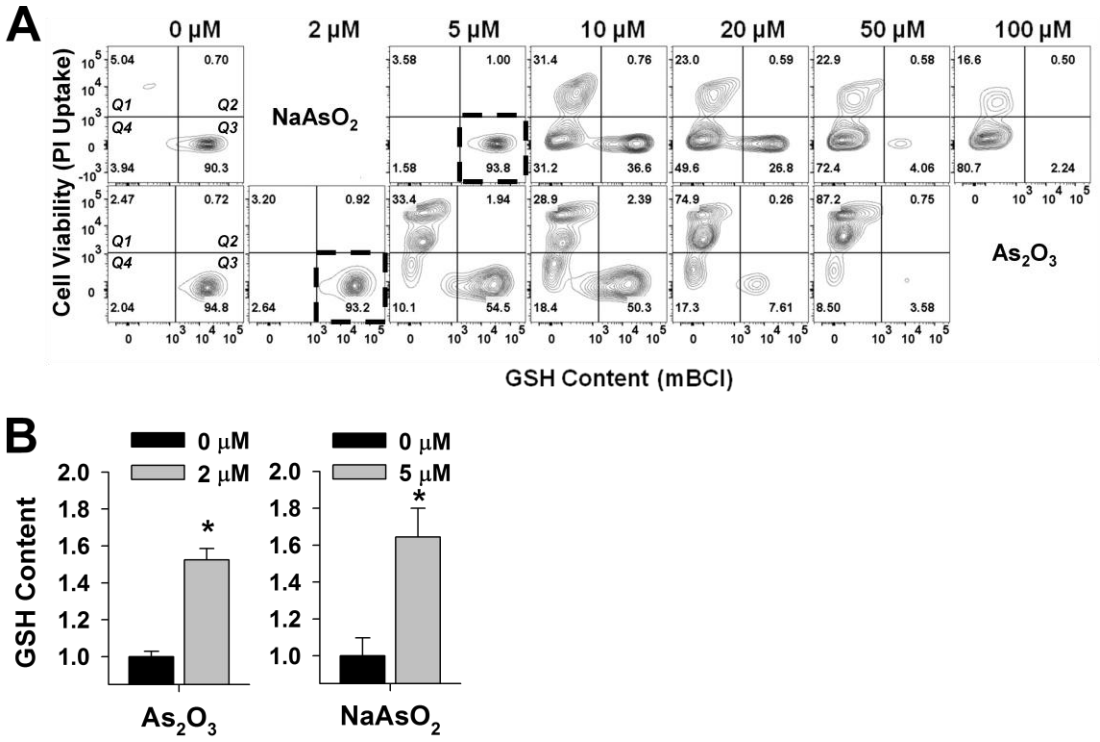


Figure 4.1: Intracellular REDOX state of Astrocytes exposed to Arsenic

(A) Flow cytometry performed on astrocytes following 48hrs of the indicated arsenic treatment. **(B)** Quantification of the fold change in mBCl signal in the dashed boxes of (A). ***:** $p<.05$ by student T-test.

While cysteine was not sufficiently labeled from U-^{13}C-glucose to detect by NMR, and glycine remained relatively constant, intracellular glutamate concentrations did decrease significantly at higher arsenic concentrations.

Additionally, the culture media was analyzed for the presence of ^{13}C-labeled products. As shown in Figure 4.2B, the efflux of both ^{13}C-labeled lactate and citrate significantly increased at low levels of arsenic exposure, while being reduced at high arsenic concentrations. Unexpectedly, the extracellular concentration of labeled glutamate, not glutamine, dramatically increased during arsenic exposure. This contrasts with the intracellular data, but indicates that glucose carbons may be shuttled to glutamate production and exported by some undetermined process.

4.3.3 Astrocyte Viability is Limited by GSH Synthesis, not GSH Recycling, during Arsenic Exposure

Due to ROS being a hallmark of arsenic exposure, and the incompletely defined role of GSH in arsenic detoxification, it was important to address whether the survival limiting aspect is GSH *de novo* synthesis or REDOX cycling of an existing GSH pool. If GSH is being used principally in a consumptive process during arsenic exposure, such as in forming a stable complex with arsenic and being excreted, inhibition of *de novo* GSH synthesis by buthionine sulphoximine (BSO) would greatly sensitize astrocytes to arsenic. However if GSH was instead being principally used as a reducing agent for arsenic derived ROS, then inhibition of the pentose phosphate pathway (PPP) by 6-aminonicotinamide (6-AN) would be expected to sensitize astrocytes to arsenic by limiting the ability to reduce oxidized glutathione (GSSG) to GSH (**Figure 4.3C**). While 6-AN had no significant effect on cell viability (**Figure 4.3A**), BSO dramatically sensitized the astrocytes to arsenic while having minor effects on viability when administered alone (**Figure 4.3B**). Both compounds were also tested against hydrogen peroxide treatment, demonstrating that each compound reduced cell viability in the presence of reactive

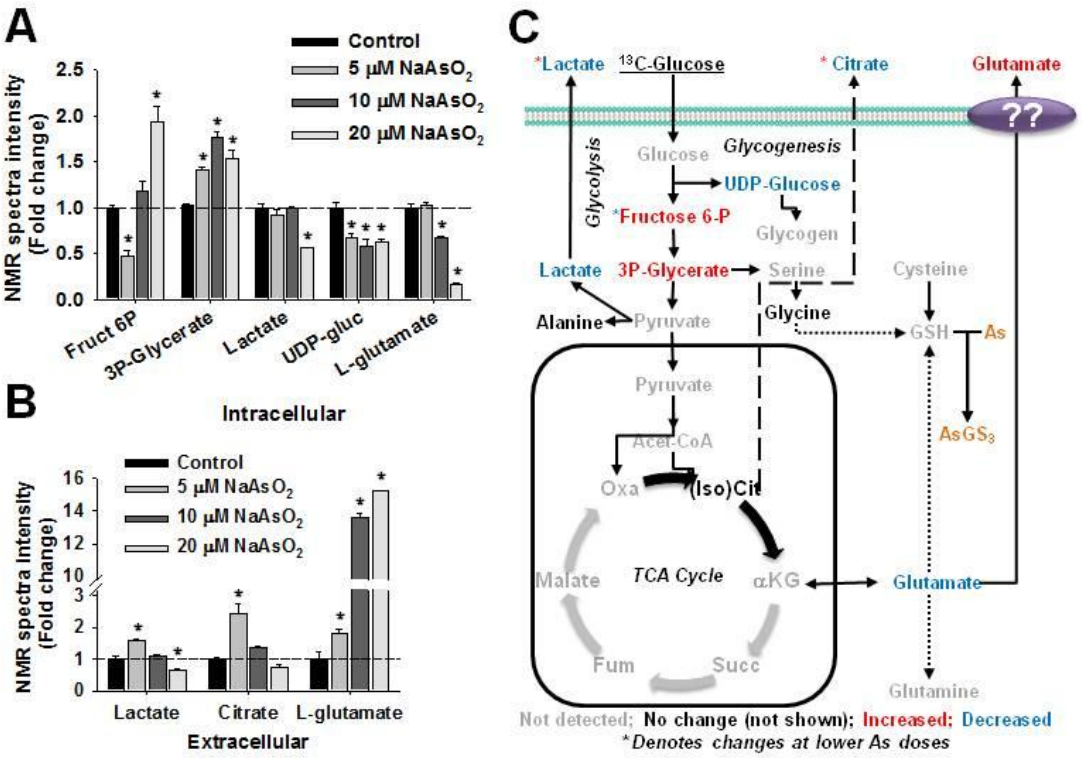


Figure 4.2: 2D-NMR Metabolomics with U-^{13}C-glucose

2D-NMR (^{1}H-^{13}C) metabolomics performed on astrocytes treated with the indicated arsenic concentrations in media containing 17.5mM U-^{13}C-glucose for 24hrs. Cellular **(A)** and media **(B)** components were measured for each sample and represented as fold change compared to control. **(C)** Pathway model of the results from (A-B). *: $p<.05$ by One Way ANOVA compared to untreated control.

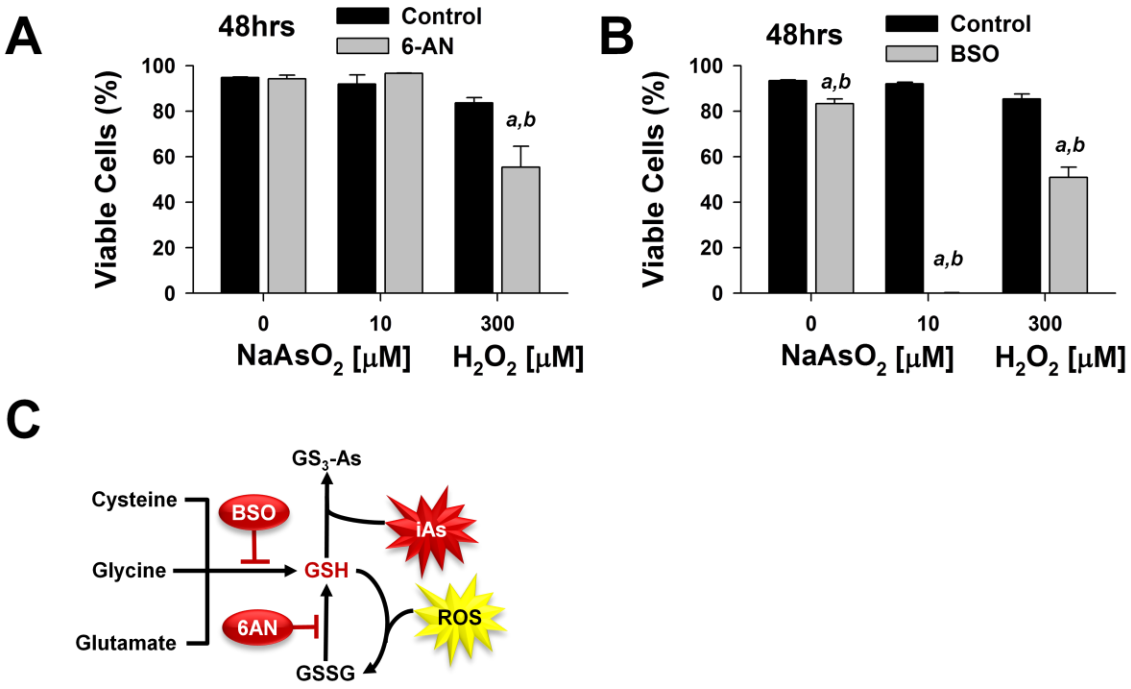


Figure 4.3: Inhibition of GSH synthesis and REDOX cycling during Arsenite Exposure

(A-B) Viable cell population determined by flow cytometry following 48hrs of sodium arsenite or hydrogen peroxide, vs. 1mM 6-AN (A) or .5mM BSO (B). **(C)** Graphical representation of GSH pathways and the inhibitors used. ***a,b***: $p<.05$ Two Way ANOVA vs. the untreated control (*a*) or the control at the same arsenic/peroxide concentration (*b*).

oxygen species (ROS) (**Figure 4.3A-B**). These results provide evidence that arsenic detoxification involves a process that consumes GSH, and implies that glutathionylation of arsenic and/or GSH efflux is a significant effect in astrocytes during arsenic exposure.

4.3.4 Astrocyte Arsenic Detoxification is Dependent upon MRP1

The accumulation of intracellular arsenic within astrocytes was determined in order to approximate a tipping point for detoxification. Utilizing inductively coupled plasma mass spectrometry (ICP-MS) a time course of intracellular arsenic accumulation in astrocytes was established for 5, 10, and 20 µM sodium arsenite ($NaAsO_2$) exposure over a 24hr period. 10 µM sodium arsenite ($NaAsO_2$) established an approximate equilibrium between efflux and influx (**Figure 4.4A**), while 5 µM sodium arsenite did not show significant arsenic accumulation beyond the 6hr time point. As MRP1 has been previously implicated in the efflux of glutathionylated arsenicals (Leslie et al., 2004), astrocyte viability and intracellular arsenic accumulation was measured in the presence of an MRP1 inhibitor (MK571). In contrast with previous results (Tadepalle et al., 2014), MK571 sensitized astrocytes to arsenite (**Figure 4.4B**), though the increase in intracellular arsenic accumulation (**Figure 4.4C**) is consistent with that study. MK571 was not found to have an effect on viability during peroxide treatment (**Appendix Figure 1**), reinforcing that MRP1 activity during arsenic exposure is not directly related to ROS production.

4.3.5 Extracellular Glutamate Accumulates in Astrocyte Cultures Exposed to Arsenic

To corroborate the glutamate efflux in the NMR results, an enzymatic assay (Glutamate-Glo, Promega) was performed to quantify the total glutamate in the astrocyte culture media. As the assay measures total glutamate, and not just glutamate produced by astrocytes, Neurobasal A media rather than DMEM was used following astrocyte confluency, as the DMEM media

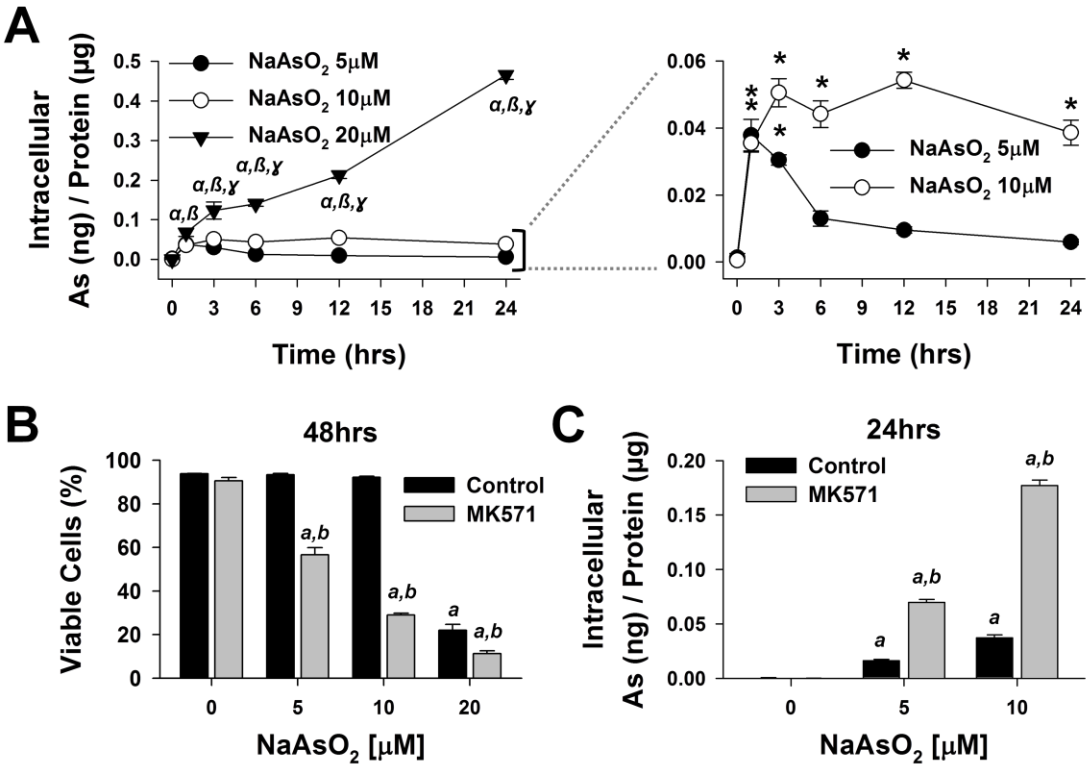


Figure 4.4: ICP-MS Time Course of Intracellular Arsenic and MRP1 Inhibition

(A) Intracellular arsenic accumulation time course by ICP-MS at the indicated arsenic exposures. ***α,ß,γ***: p<.05 by Two Way ANOVA within a time point between: 20µM vs. 10µM (*α*), 20µM vs. 5µM (*ß*), and 10µM vs. 5µM (*γ*). *****: p<.05 by Two Way ANOVA between untreated cells and treated cells. **(B)** Viable cell population determined by flow cytometry following 48hrs of sodium arsenite vs. 25µM MK571. **(C)** Intracellular arsenic accumulation by ICP-MS after 24hrs at the indicated arsenic exposures vs. 25µM MK571. ***a,b***: p<.05 Two Way ANOVA vs. the untreated control (*a*) or the control at the same arsenic concentration (*b*).

contained approximately 50 µM glutamate. As in the NMR experiment, the presence of arsenic dramatically increased the quantity of glutamate produced by astrocytes (**Figure 4.5A**).

4.3.6 Extracellular Glutamate Accumulation is not dependent on MRP1 or ɣGT

As described in chapter 2, astrocytes produce GSH for neurons, which is broken down by astrocyte ɣ-glutamyltranspeptidase (ɣGT) and neuronal aminopeptidase N (ApN) into its amino acid components. Additionally, this astrocyte GSH export process and arsenic detoxification are both dependent upon MRP1 (**Figure 4.4B-C**). Enzymatic quantification of extracellular glutamate from astrocytes given ɣGT (Acivicin) or MRP1 (MK571) inhibitors was used to determine if the decomposition of arsenic-GSH complexes or GSH were the sources of the extracellular glutamate, but no significant differences were observed (**Figure 4.5A**).

4.3.7 Extracellular Glutamate Accumulates Independently of Arsenic during EAAT1/2 Inhibition, but only EAAT1 sensitizes Astrocytes to Arsenic

As excitatory amino acid transporters have been reported to reverse transport under certain conditions, inhibitors UCPH-101 (EAAT1) and WAY-213613 (EAAT2) were applied to astrocytes with or without arsenic, and found that significant glutamate accumulated outside of the cell independent of arsenic exposure. (**Figure 4.5A**). Given that intracellular glutamate decreased in the presence of arsenic (**Figure 4.2A**) and that EAAT1/2 inhibition increased extracellular glutamate accumulation (**Figure 4.5A**), it was important to determine if the function of EAAT1 and EAAT2 would sensitize astrocytes to arsenic. Administration of both UCPH-101 and WAY-213613 caused a significant decrease in viability in the presence of arsenic (**Figure 4.5B**). To test if this was a synergistic effect of both compounds or if one transporter was primarily responsible, the test was repeated with each inhibitor assayed independently, and

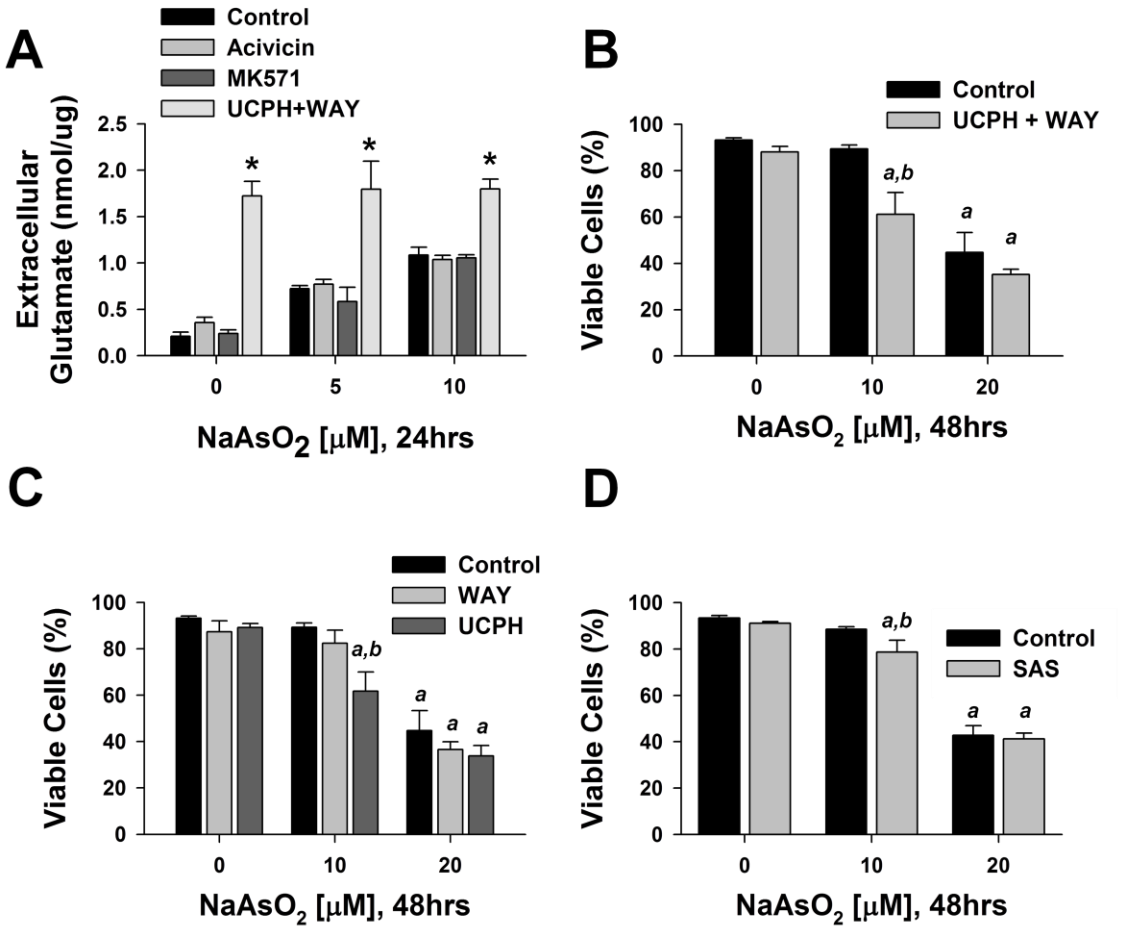


Figure 4.5: Extracellular Glutamate and Arsenic Sensitivity Impacted by EAAT Inhibition

(A) Extracellular glutamate concentration measured by Glutamate-Glo following arsenic treatment vs. 100μM acivicin, 25μM MK571, or a mixture of 10μM UCPH-101 and 10μM WAY213613. **(B-D)** Viable cell population determined by flow cytometry following 48hrs of sodium arsenite vs. (B) a mixture of 10μM UCPH-101 and 10μM WAY213613, (C) 10μM UCPH-101, (C) 10μM WAY213613, or (D) 50μM SAS. *****: $p<.05$ by Two Way ANOVA compared to the control at the same arsenic concentration. ***a,b***: $p<.05$ Two Way ANOVA vs. the untreated control (*a*) or the control at the same arsenic concentration (*b*).

demonstrated that the effect was due to EAAT1, with no significant effect observed with EAAT2 inhibition (**Figure 4.5C**).

4.3.8 Inhibition of xCT has Minor Sensitizing Effects on Astrocytes to Arsenic

The cystine-glutamate antiporter (xCT) is a means to acquire the limiting component cysteine for GSH synthesis in astrocytes through glutamate efflux(Cho and Bannai, 1990;Allen et al., 2001;Allen et al., 2002;Seib et al., 2011). The increased extracellular glutamate accumulation (**Figure 4.5A**) and the necessity of GSH synthesis (**Figure 4.3B**) during arsenic exposure in astrocytes implies the xCT system may be required to maintain intracellular cysteine concentrations during arsenic exposure. However, only a small but significant increase in arsenic sensitivity was observed with the application of sulfasalazine (SAS) during arsenic exposure, indicating that xCT is not likely a critical factor (**Figure 4.5D**). Additionally, astrocyte viability was tested during arsenic exposure in media supplemented with cystine (200µM) or methionine (200µM), but no protective or sensitizing effect was observed (**Appendix Figure 2A**)

4.3.9 Inhibition of Mitochondria or Transamination Sensitizes Astrocytes to Arsenic

While the route of glutamate efflux remains to be determined, the observed reduction of intracellular glucose labeled glutamate during arsenic exposure (**Figure 4.2A**) coupled with the arsenic sensitizing effect of EAAT1 inhibition (**Figure 4.5D**) implies that glutamate could be a limiting substance for astrocyte survival. Though the most direct route of glutamate procurement would be the deamination of glutamine through glutaminase, preliminary results have shown that the application of the glutaminase inhibitor BPTES had no significant effect on cell viability with and without the presence of arsenic (Suppl. Figure 2B). *De novo* synthesis of glutamate requires mitochondrial processes, and astrocytes were therefore challenged with mitotoxins to determine if mitochondrial inhibition altered arsenic sensitivity. In agreement with previous results (Rathinam

et al., 2012;Zhang et al., 2015), astrocytes remained viable in the presence of low concentrations of the mitochondrial toxins rotenone, paraquat (PQ), or 1-methyl-4-phenylpyridinium (MPP^+), but each of these compounds sensitize astrocytes to arsenic (**Figure 4.6A**). Experiments were performed to determine whether electron transport chain or carbon input restrictions to the mitochondria impacted total ATP. ATP levels exhibited small but significant decreases when arsenic was co-administered with UK5099, etomoxir, or rotenone (**Figure 4.6B**). Carbon restriction to the mitochondria similarly sensitized the astrocytes to arsenic, while having no significant independent effect (**Figure 4.6C**). Additionally, astrocyte viability was tested in the presence of aminooxyacetic acid (AOAA), as transamination is a key component of glutamate synthesis. A similar arsenic sensitization occurred with AOAA (**Figure 4.6C**). 24hr time point ICP-MS experiments were then performed to determine if UK5099, etomoxir, or AOAA affected arsenic accumulation in astrocytes. While each compound increased arsenic accumulation, surprisingly, no significant effect was observed except for etomoxir (**Figure 4.6D**). Similarly, measurements of extracellular glutamate following compound treatments did not yield any significant changes compared to controls, except for etomoxir (**Figure 4.6E**).

4.3.10 Etomoxir and Arsenic Co-administration Damages Mitochondrial Respiratory Capacity

Given that the mitochondria is providing a critical function in astrocyte survival during arsenic exposure (**Figure 4.6A**) and that etomoxir induces significant effects during arsenic exposure in total ATP (**Figure 4.6B**), viability (**Figure 4.6C**), and intracellular arsenic accumulation (**Figure 4.6D**), mitochondrial respiration was measured during arsenic and/or etomoxir exposure utilizing the Seahorse Mito Stress Test (Agilent). As shown in Figure 6, arsenic had minimal effects on mitochondrial respiration, with a small yet significant decrease in maximal respiration. Etomoxir treatment almost completely stopped astrocyte mitochondrial respiration, but only a small change in maximal respiration was observed. Thus, mitochondrial

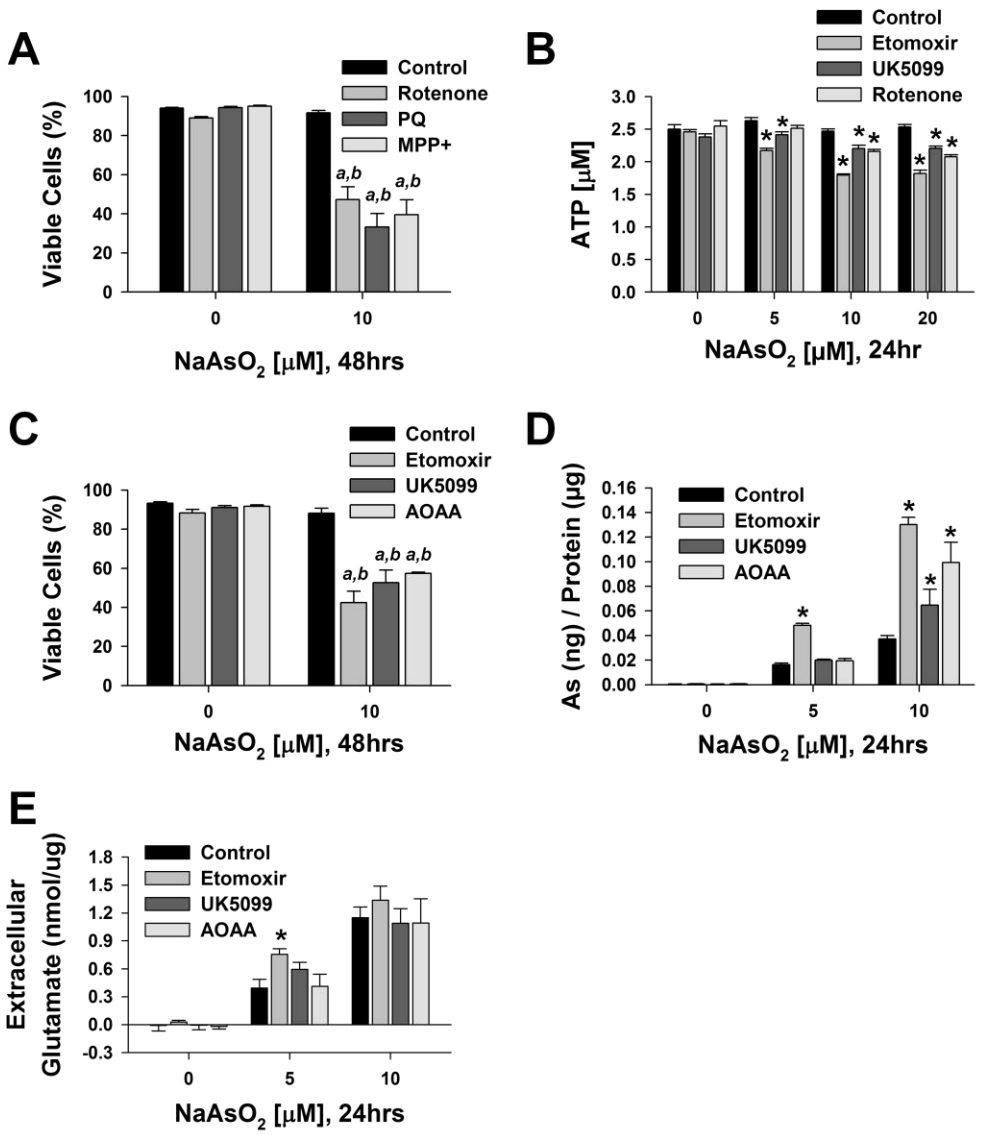


Figure 4.6: Mitochondrial Functions are Essential During Arsenic Exposure

(A) Viable cell population determined by flow cytometry following 48hrs of sodium arsenite vs. 1µM rotenone, 50µM paraquat, or 2.5mM MPP^+. **(B)** ATP quantified by Cell-Titer Glo with the indicated arsenic concentrations vs. 200µM etomoxir, 5µM UK5099, or 1µM rotenone.

(Figure 4.6: Mitochondrial Functions are Essential During Arsenic Exposure continued)

(C-E) Viable cell population determined by flow cytometry (C), intracellular arsenic accumulation by ICP-MS (D), and extracellular glutamate accumulation measured by Glutamate-Glo (E) at the indicated arsenic exposures vs. 200μM etomoxir, 5μM UK5099, or 5mM AOAA. *****: $p<.05$ by Two Way ANOVA compared to the control at the same arsenic concentration. ***a,b***: $p<.05$ Two Way ANOVA vs. the untreated control (*a*) or the control at the same arsenic concentration (*b*).

electron transport machinery is still intact in the presence of etomoxir. However, co-administration of etomoxir with arsenic led to a cessation of mitochondrial respiration and a dramatic decrease in maximal respiration. The results imply that the electron transport capabilities of the mitochondria were effectively disabled by the co-treatment of arsenic and etomoxir.

4.3.11 $^{13}C/^{15}N$ Glutamate NMR Preliminary: Arsenic Doesn't Alter GSH Incorporation, but Increases Extracellular Glutamate and Intracellular Glutamine.

Given that glutamate is being synthesized by glucose precursors (**Figure 4.2**) and effluxed to the extracellular space (**Figure 4.5A and 4.6E**), NMR samples were generated using U-$^{13}C/^{15}N$ glutamate to determine if glutamate metabolism had been altered by the presence of arsenic. While the final data analysis is still pending, preliminary data indicate that glutamate carbons are being incorporated into lactate, glutamine, and GSH. However, the changes observed with arsenic treatment were an increase in extracellular glutamate and intracellular glutamine, with no observed changes in extracellular lactate or intracellular GSH. (**Appendix Figure 3**).

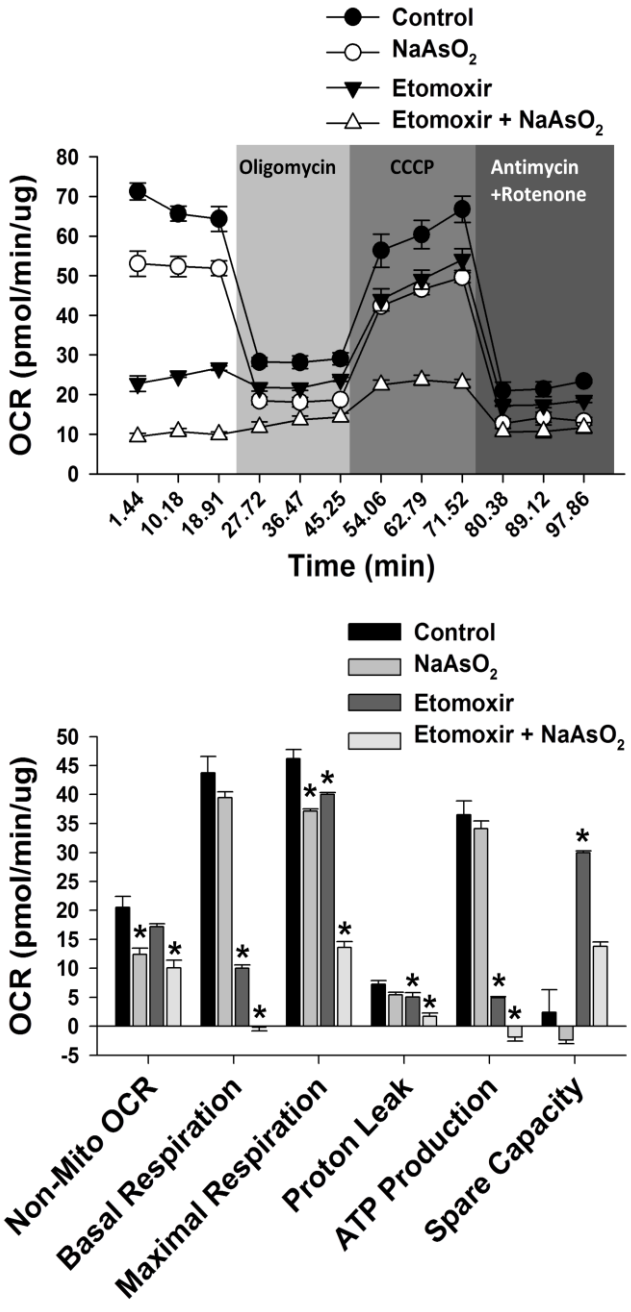


Figure 4.7: Mito Stress Test during Arsenic and Etomoxir Exposure

Seahorse Mito Stress Test performed in an XFe24 Analyzer. Astrocytes were treated with/without 200µM etomoxir and/or 10µM arsenite for 24hrs. Final well concentrations of applied inhibitors are 1µM oligomycin, 1µM CCCP, and a mixture of .5µM rotenone and .5µM antimycin A. *: $p<.05$ by Two Way ANOVA compared to control.

4.4 Discussion

4.4.1 Astrocyte Arsenic Detoxification is Dependent upon GSH Biosynthesis and MRP1

The preliminary data from the Dr Annadurai Anandhan indicated that lethal arsenic exposure causes GSH depletion, and that sub-lethal arsenic exposure increases the intracellular GSH content, in agreement with previous observations (Sagara et al., 1996;Koehler et al., 2014). Whether this loss of intracellular GSH was principally due to a net loss of intracellular GSH, or was being oxidized in a transitory, recyclable process remained to be determined. With 6-AN having a minimal effect, and BSO having such a dramatic effect on astrocyte viability, we are confident that GSH is being lost in a process related to arsenic exposure, either by formation of an As-GS_X complex and/or by the direct efflux of GSH from the astrocytes.

While As-GS_X complex efflux has not been directly demonstrated in astrocytes to our knowledge, both GSH efflux (Hirrlinger et al., 2002;Hirrlinger and Dringen, 2005) and arsenic induced GSH efflux (Tadepalle et al., 2014) have been linked to MRP1 in astrocytes. We did not observe an increase in intracellular GSH with MRP1 inhibition (*data not shown*). The use of a time-course ICP-MS to quantify intracellular arsenic accumulation during arsenic exposure with and without MK571 provided two important pieces of information: (1) sodium arsenite concentrations can be divided into sub-lethal (5 μM), steady state (10 μM), and lethal (20 μM) dosages, and (2) reinforces that MRP1 inhibition directly increased arsenic accumulation within the astrocytes and sensitizes astrocytes to arsenic. This information allowed us to narrow subsequent experiments around the 10 μM exposure, and provided further evidence that MRP1 is directly or indirectly involved in the export of arsenic and not just GSH export for neuronal use. The increased arsenic accumulation with MK571 had been previously reported, but the prior study performed the measurement after a two hour exposure (Tadepalle et al., 2014), which our data indicates is within an acute response phase of arsenic exposure. We believe our results strengthen prior observations, and provide a foundation for the exploration of arsenic

accumulation at the later, chronic stages of exposure. Combined with the BSO/6-AN data, we believe that a glutathionylated arsenical, or an arsenical that relies on GSH as a co-substrate, is exported in an MRP1 dependent process.

4.4.2 Arsenic Exposure Induces Excitotoxic Glutamate Accumulation in Astrocyte Cultures

To our knowledge, a metabolomic profile comparison of primary cortical astrocytes with variable arsenic exposure has not been performed. Tracking the changes in carbon labeling allowed insights into how metabolism shifted in response to arsenic toxicity. Intracellular glutamate labeling depleted with increasing arsenic, while the opposite was observed extracellularly (**Figure 4.2B** and **Figure 4.5A**). Importantly, ^{13}C-labeled glutamate was distinguishable from glutamine, which is the expected product of astrocytes for neurotransmitter recycling. The finding that glutamate was significantly produced from a glucose carbon source and was exported has profound implications for neurotransmission and neuronal viability. The NMR experiments only provided estimates of relative metabolite concentration changes.

Validation of the NMR glutamate efflux was performed by the Glutamate-Glo enzymatic kit, where arsenic treated astrocytes demonstrated a very high rate of glutamate production. As the DMEM:f12 media used in our experiments had ~50 µM of glutamate as part of its formulation (validated with a preliminary test of the DMEM:f12 media with Glutamate-Glo, *data not shown*), we utilized Neurobasal A medium supplemented with B27 and 2.5 mM glutamine (to match DMEM:f12 glutamine) in order to quantify astrocyte produced glutamate. Our results contrast with a previous report that found an approximate 1.5 µM increase in extracellular glutamate after a 24hr exposure of 10 µM sodium arsenite to primary cortical astrocyte cultures (Wang et al., 2012). At the same arsenic exposure, we repeatedly observed over 30 µM glutamate within the media. In fact, an exposure of only 5 µM of sodium arsenite yielded ~ 20 µM glutamate in the media. A Glutamate-Glo experiment was also performed with astrocytes in our

DMEM:f12 media (*data not shown*), showing that a 10 µM sodium arsenite treatment produced an additional 25 µM of glutamate over background. The extracellular space within the CNS has been measured to contain ~0.6 µM glutamate. Furthermore, excitotoxic effects have been observed in neurons cultured independently of astrocytes or in brain regions, like the hippocampus, at an extracellular glutamate concentration of 2-5 µM (Lipton and Rosenberg, 1994;Mark et al., 2001;Chen and Lipton, 2006). Thus, our results have profound implications for neuronal viability.

Initially, the extracellular glutamate was believed to be derived from the GSH or As-GSX complexes that efflux from MRP1, as ɣGT has been described as a means to recycle glutamate for GSH resynthesis in cancer cells during drug treatment (Rajpert-De Meyts et al., 1992;Hochwald et al., 1996;Pompella et al., 2006;Ramsay and Dilda, 2014). But, the Glutamate-Glo results for acivicin and MK571 indicate that GSH or As-GSX are not the source (**Figure 4.5A**). One limitation of the acivicin experiment (**Figure 4.5A**) is the lack of a positive control to ensure its functionality. Nevertheless, it stands to reason that arsenic induced extracellular glutamate accumulation is due to a deficiency of astrocyte glutamate reuptake, and/or an induction of glutamate efflux that is not dependent on MRP1/ ɣGT.

Two principal glutamate reuptake transporters in astrocytes are EAAT1/2, with a high localization near the synaptic cleft and capillary processes (Roberts et al., 2014), and the reversal of glutamate transport during membrane depolarization (Szatkowski et al., 1990). Crystal structures have shown that UCPH-101 inhibits EAAT1 allosterically by preventing the substrate translocation transition state (Canul-Tec et al., 2017), while WAY213613 is a competitive inhibitor of EAAT2 (Dunlop et al., 2005). Our results indicate that extracellular glutamate accumulation dramatically increases in the presence of EAAT1/2 inhibition (**Figure 4.5B**). Interestingly, extracellular glutamate significantly accumulated with EAAT1/2 inhibition independent of arsenic, which implies the process that causes glutamate loss in astrocytes is

active in a non-stress state. Furthermore, the results suggest that either EAAT1/2 counteracted this process or the inhibitors reverse glutamate transport in astrocytes.

Another source of glutamate extrusion is the cystine/glutamate antiporter xCT. Cysteine has been described as the limiting substrate of GSH synthesis in many cell types, including astrocytes (Wang and Cynader, 2000;McBean, 2017). Astrocytes have also been found to preferentially utilize cystine over cysteine for GSH synthesis (Kranich et al., 1996). Accordingly, xCT may increase its activity to export glutamate to acquire cystine for this process. As shown in Figure 4.5D, SAS has a minor but significant sensitizing effect to arsenic in astrocytes. We also demonstrated that increasing the extracellular cystine or methionine concentrations did not alter astrocyte sensitivity to arsenic (**Appendix Figure 2A**).

4.4.3 Mitochondrial Function is Integral to Astrocyte Viability in the Presence of Arsenic

While our results do not reveal the means of glutamate efflux, the data strongly demonstrate a role for mitochondrial metabolism during arsenic exposure in astrocytes. The NMR experiments revealed the increased integration of glucose carbons into citrate and glutamate pools (**Figure 4.2**), which suggested mitochondrial metabolism must be involved. However, whether the increased glucose carbon integration into metabolic pathways for citrate and glutamate was serving a critical function during arsenic exposure remained unknown. We demonstrated that mitochondrial toxins (PQ) and complex I inhibition (Rotenone, MPP^+) sensitized astrocytes to arsenic, and that astrocytes remained viable in the presence of these inhibitors in the absence of arsenic (**Figure 4.6A**). A previous study demonstrate that astrocytes are not dependent on *Cox10* in an in vivo model (Supplie et al., 2017). Thus, we do not believe this sensitization is due to a deficiency in OXPHOS derived ATP. Co-administration of rotenone and 10/20 μM arsenic did result in a net decrease of 20% in the total ATP, though no differences were observed with either

independent treatment (**Figure 4.6B**). We can also conclude that pyruvate carbon entry into the mitochondria is required during arsenic exposure in astrocytes (**Figure 4.6C**).

While it is clear that the mitochondria perform an integral function during arsenic exposure, the precise role cannot be definitively determined by our data. Nevertheless, our observations do allow for identifying potential mitochondrial functions critical for astrocytes during arsenic exposure.

One possible response to arsenic exposure is the use of complex I to balance the NAD+/NADH ratio in the cytosol. Cancer cells have long been known to metabolize glucose to lactate (Warburg, 1956), and have demonstrated that their mitochondria were not required for ATP production (King and Attardi, 1989). The cancer cell proliferation reduction caused by oligomycin (an inhibitor of ATP synthetase) was shown to be reversed by low doses of the uncoupling agent FCCP (Sullivan et al., 2015). Additionally, cancer cells treated with complex I/III inhibitors (phenformin + piericidin/antimycin) demonstrated a metabolic shift that was dependent on exogenous pyruvate and a reversal of MDH1 and GOT1 directionality toward the production of aspartate (Birsoy et al., 2015). These experiments illustrated that NAD+/NADH balancing was a key mitochondrial function in cancer cells. FCCP allowed ETC activity to resume by removing mitochondrial hyper-polarization and exogenous pyruvate converting to lactate bioenergetically balanced the reductive MDH1/GOT1 path of aspartate synthesis. In a similar fashion, astrocytes may require complex I to regenerate NAD+ from NADH, as the synthesis of □-ketoglutarate from acetyl-CoA and oxaloacetate in the oxidative arm of the TCA cycle generates NADH from NAD+. Additionally, while the full conversion of glucose to lactate does not perturb the NAD+/NADH ratio, our NMR data demonstrate that some proportion of glucose carbons become glutamate, changing the NAD+/NADH ratio by reducing NAD+ to NADH via isocitrate dehydrogenase. Therefore, complex I in the mitochondria of astrocytes may

primarily act as an electron sink during arsenic exposure in order to maintain the NAD+/NADH ratio by oxidizing NADH to NAD+ rather than to generate ATP.

The loss of viability during arsenic exposure when administering UK5099 or etomoxir indicates that carbon entry into the mitochondria is vital. Unlike cancer cell studies (Birsoy et al., 2015;Sullivan et al., 2015), glutamate rather than aspartate seems to be the destination for the pyruvate carbons entering the mitochondria. Citrate has been previously observed as an alternative carbon entry into the mitochondria for astrocytes (McKenna et al., 1995;Vogel et al., 1998)) (**Figure 3.1** and **3.2**). Besides Seahorse related assays, very few studies have applied UK5099 to astrocytes, presumably because these prior studies suggest that UK5099 does not have an effect on astrocytes (Divakaruni et al., 2017;Weightman Potter et al., 2019). Nevertheless, applications of UK5099 to other cell types have successfully highlighted the compensatory mechanisms utilized for carbon entry into the mitochondria. These compensatory mechanisms include upregulation of fatty acid oxidation (Vacanti et al., 2014), an increase in glutamate/glutamine metabolism (Vacanti et al., 2014;Yang et al., 2014;Gray et al., 2015), and pyruvate-alanine interconversion (McCommis et al., 2015). However, the cell types were not glycolytic or were highly proliferative, which is unlike astrocytes. Additionally, astrocytes are sensitive to ammonia toxicity caused by mitochondrial glutamine metabolism (Pichili et al., 2007). Our preliminary BPTES results also indicate that glutaminase is not a vital process (**Appendix Figure 2B**). It is possible that by restricting a single mitochondrial carbon source, partial compensation could be achieved by upregulating the other mitochondrial carbon import pathways. This could be why the UK5099, etomoxir, and AOAA results had nearly equivalent effects on cell viability: the loss of one carbon source is compensated by the other sources.

The effect of etomoxir could be an indicator that fatty acid oxidation (FAO) is vital. FAO is purported to be the source of acetyl-CoA, has already been demonstrated to be active in astrocytes (Edmond et al., 1987;Ebert et al., 2003), and astrocytes restrict pyruvate

dehydrogenase complex activity with extensive phosphorylation (Halim et al., 2010). FAO produces significant amounts of NADH and $FADH_2$, which creates a need for an electron sink in order to maintain NAD+/NADH and FAD/$FADH_2$ bioenergetic balancing. The role of the mitochondrial electron transport chain during arsenic exposure may be to maintain this bioenergetics balance. To further explore this hypothesis, we utilized Seahorse Mito Stress Test to determine the oxygen consumption rate of astrocyte mitochondria (**Figure 4.7**). In the presence of etomoxir and independent of arsenic, we observed minimal basal oxygen consumption and a minimal effect of oligomycin treatment. However, application of CCCP demonstrated that the electron transport chain was still functional and at a level similar to control cells. One possible interpretation is that the ETC normally acts to balance the bioenergetic disturbance of FAO, but the removal of mitochondrial FAO effectively eliminates the majority of the ETC's purpose. However, arsenic exposure requires a mitochondrial process that uses glucose and fatty acid carbon inputs. Thus, the loss of FAO leads to the observed reduction in viability.

Inhibition of transamination processes by AOAA causes a similar loss in viability during arsenic exposure as seen with UK5099 and etomoxir. However, transamination participates in both bioenergetic balancing and biosynthesis, and is not restricted to mitochondrial processes. Previous reports on astrocytes have shown that AOAA restricts the metabolism and synthesis of glutamate from TCA cycle intermediates (Westergaard et al., 1996;Sonnewald et al., 1997). Further studies highlighted that glutamine synthesis required aspartate (Pardo et al., 2011), further reinforcing the potential role of transamination for glutamate synthesis (McKenna et al., 2016). On the other hand, astrocytes upregulate glucose and lactate metabolism in the presence of 5 mM AOAA, indicating that the malate-aspartate shuttle is not the mechanism of electron balance in astrocytes (McKenna et al., 1993). This observation doesn't outright disprove the hypothesis that the ETC is being used for bioenergetic balancing. The glycerol 3-phosphate shuttle has been implicated in astrocytes (McKenna et al., 1993;Waagepetersen et al., 2001), though more

verification is required due to discrepancies in the shuttle's expression in astrocytes (McKenna et al., 2006). This argues in favor of our AOAA results being related to aminotransferase inhibition in regards to glutamate synthesis, and, therefore; that *de novo* glutamate synthesis is crucial for astrocyte viability. However, we did not observe a decrease in extracellular glutamate accumulation in the presence of AOAA (**Figure 4.6E**), which argues against this idea, but we did observe that AOAA increased arsenic accumulation within astrocytes (**Figure 4.6D**). This result indicates that a transamination process is involved in arsenic detoxification.

While the glucose NMR results (**Figure 4.2**) require further analysis and supplemental experimentation to make definitive conclusions, we can speculate as to what the currently observed metabolite trends indicate. The reduction in UDP-glucose at all arsenic concentrations is an indication that glycogenesis is being downregulated in the presence of arsenic. This is supported by previous studies that described glucose consumption and lactate production to increase during acute arsenic toxicity (Tadepalle et al., 2014). While the intracellular lactate level did not significantly change (with the exception of the highest arsenic concentrations tested), we observed an increase in the extracellular lactate excretion at sublethal arsenic exposure. Extracellular citrate followed a similar trend as lactate, indicating that increased glucose utilization is feeding both cytosolic and mitochondrial pathways. Fructose-6-phosphate decreased at sublethal arsenic levels, remained equivalent at equilibrium concentrations, and rose at lethal doses. Conversely, 3-phosphoglycerate levels increased for all arsenic concentrations tested (**Figure 4.2A**). The 6-AN viability results (**Figure 4.3A**) suggest cytosolic NADPH is not a limiting resource during arsenic exposure. Metabolomic studies in other cell types studying ROS and PPP induction found that upper glycolysis intermediates (such as fructose-6-phosphate) increase, while lower glycolysis intermediates (such as 3-phosphoglycerate) decrease (Kuehne et al., 2015;Lucarelli et al., 2015). Since sublethal arsenic exposure exhibits an opposite trend and we did not identify changes in PPP intermediates, glycolysis may be preferred over PPP during

arsenic toxicity. However, that does not explain why the fructose-6-phosphate trend reverses at lethal arsenic concentrations.

Interestingly, the preliminary glutamate NMR results (**Appendix Figure 3**) show that extracellular glutamate levels increased during arsenic exposure, which corroborates our findings from the glucose NMR (**Figure 4.2**) and the Glutamate-Glo experiments (**Figure 4.5A** and **Figure 4.6E**). The extracellular lactate levels did not change, indicating that glutamate metabolism did not change. Surprisingly, GSH levels did not increase with arsenic, which we would have expected given the requirement for GSH synthesis during arsenic exposure (**Figure 4.3B**). It is possible that glutamate is not a limiting reagent for GSH synthesis (that it is cysteine instead), and that glutamate is being utilized for another, undefined process in astrocytes during arsenic exposure.

4.5 Conclusions

We can conclusively demonstrate that arsenic detoxification in astrocytes requires GSH synthesis and MRP1. We also demonstrate that in the presence of arsenic, astrocytes synthesize glutamate from glucose precursors and efflux glutamate into the extracellular space to concentrations beyond what is considered the excitotoxicity threshold for most glutamatergic neurons. We further show that inhibition of EAAT1/2 causes an increase in extracellular glutamate independently of arsenic, and that EAAT1, but not EAAT2, inhibition sensitizes astrocytes to arsenic. Additionally, we demonstrate that the mitochondria perform a critical, yet undefined, function in astrocytes exposed to arsenic, and that inhibition of the mitochondrial pyruvate carrier or transamination processes sensitizes astrocytes to arsenic (**Figure 4.8**).

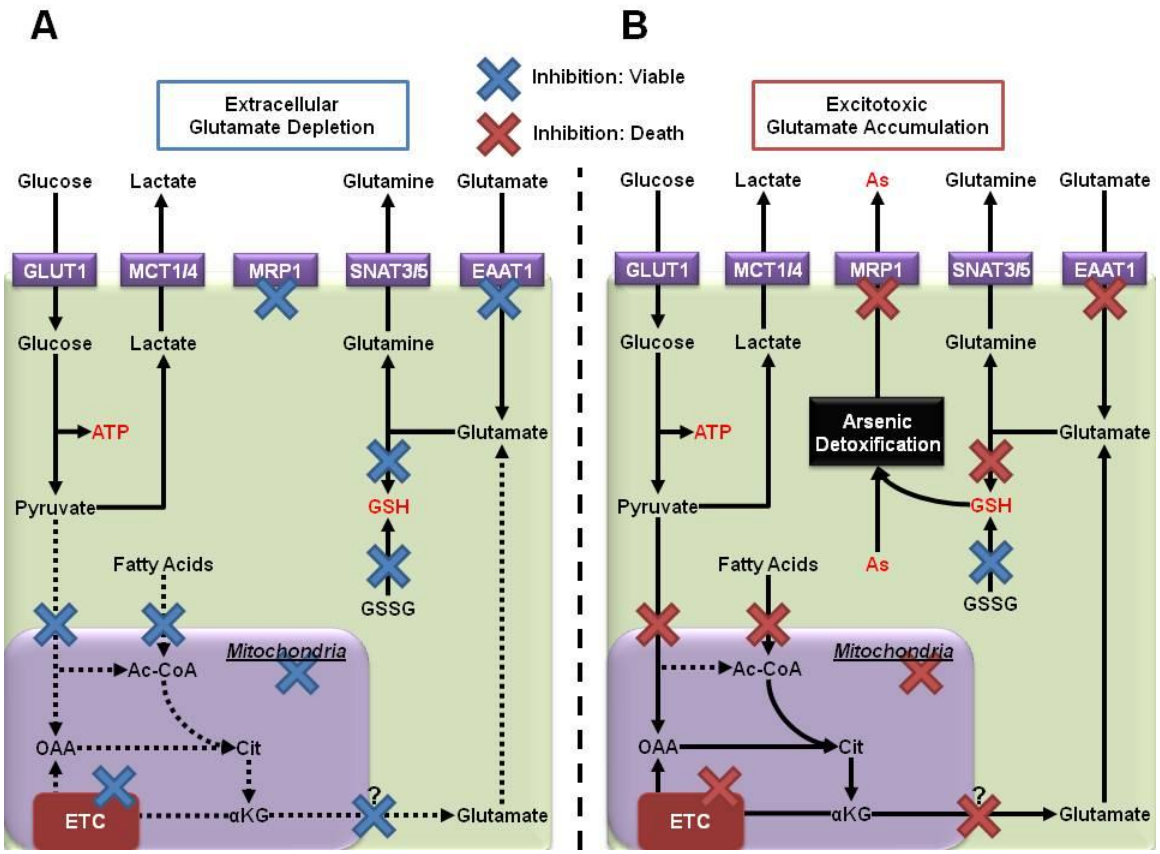


Figure 4.8: Essential Tested Metabolic Pathways during Arsenic Exposure

Model of astrocyte metabolism. **(A)** Astrocytes remain viable during chemical inhibition of mitochondrial metabolism, and mechanisms of GSH synthesis and glutamate transport. **(B)** During arsenite exposure, mitochondrial metabolism, GSH synthesis, and glutamate transport become essential for survival, in addition to the activity of MRP1. Glutamate accumulates extracellularly to excitotoxic concentrations during arsenite exposure by a yet determined mechanism. (?): The role of transamination requires further characterization, and cannot be definitely linked to glutamate synthesis.

4.6 References

Ahsan, H., Chen, Y., Parvez, F., Zablotska, L., Argos, M., Hussain, I., Momotaj, H., Levy, D., Cheng, Z., Slavkovich, V., Van Geen, A., Howe, G.R., and Graziano, J.H. (2006). Arsenic exposure from drinking water and risk of premalignant skin lesions in Bangladesh: baseline results from the Health Effects of Arsenic Longitudinal Study. *Am J Epidemiol* 163**,** 1138-1148.

Ahsan, H., Perrin, M., Rahman, A., Parvez, F., Stute, M., Zheng, Y., Milton, A.H., Brandt-Rauf, P., Van Geen, A., and Graziano, J. (2000). Associations between drinking water and urinary arsenic levels and skin lesions in Bangladesh. *J Occup Environ Med* 42**,** 1195-1201.

Allen, J.W., Shanker, G., and Aschner, M. (2001). Methylmercury inhibits the in vitro uptake of the glutathione precursor, cystine, in astrocytes, but not in neurons. *Brain Res* 894**,** 131-140.

Allen, J.W., Shanker, G., Tan, K.H., and Aschner, M. (2002). The consequences of methylmercury exposure on interactive functions between astrocytes and neurons. *Neurotoxicology* 23**,** 755-759.

Anandhan, A., Lei, S., Levytskyy, R., Pappa, A., Panayiotidis, M.I., Cerny, R.L., Khalimonchuk, O., Powers, R., and Franco, R. (2017). Glucose Metabolism and AMPK Signaling Regulate Dopaminergic Cell Death Induced by Gene (alpha-Synuclein)-Environment (Paraquat) Interactions. *Mol Neurobiol* 54**,** 3825-3842.

Anandhan, A., Rodriguez-Rocha, H., Bohovych, I., Griggs, A.M., Zavala-Flores, L., Reyes-Reyes, E.M., Seravalli, J., Stanciu, L.A., Lee, J., Rochet, J.C., Khalimonchuk, O., and Franco, R. (2015). Overexpression of alpha-synuclein at non-toxic levels increases dopaminergic cell death induced by copper exposure via modulation of protein degradation pathways. *Neurobiol Dis* 81**,** 76-92.

Banerjee, M., Kaur, G., Whitlock, B.D., Carew, M.W., Le, X.C., and Leslie, E.M. (2018). Multidrug Resistance Protein 1 (MRP1/ABCC1)-Mediated Cellular Protection and Transport of Methylated Arsenic Metabolites Differs between Human Cell Lines. *Drug Metab Dispos* 46**,** 1096-1105.

Bergquist, E.R., Fischer, R.J., Sugden, K.D., and Martin, B.D. (2009). Inhibition by methylated organo-arsenicals of the respiratory 2-oxo-acid dehydrogenases. *J Organomet Chem* 694**,** 973-980.

Bhinderwala, F., Lei, S., Woods, J., Rose, J., Marshall, D.D., Riekeberg, E., Leite, A.L., Morton, M., Dodds, E.D., Franco, R., and Powers, R. (2019). Metabolomics Analyses from Tissues in Parkinson's Disease. *Methods Mol Biol* 1996**,** 217-257.

Birsoy, K., Wang, T., Chen, W.W., Freinkman, E., Abu-Remaileh, M., and Sabatini, D.M. (2015). An Essential Role of the Mitochondrial Electron Transport Chain in Cell Proliferation Is to Enable Aspartate Synthesis. *Cell* 162**,** 540-551.

Canul-Tec, J.C., Assal, R., Cirri, E., Legrand, P., Brier, S., Chamot-Rooke, J., and Reyes, N. (2017). Structure and allosteric inhibition of excitatory amino acid transporter 1. *Nature* 544**,** 446-451.

Castro-Coronel, Y., Del Razo, L.M., Huerta, M., Hernandez-Lopez, A., Ortega, A., and Lopez-Bayghen, E. (2011). Arsenite exposure downregulates EAAT1/GLAST transporter expression in glial cells. *Toxicol Sci* 122**,** 539-550.

Chakraborti, D., Rahman, M.M., Mukherjee, A., Alauddin, M., Hassan, M., Dutta, R.N., Pati, S., Mukherjee, S.C., Roy, S., Quamruzzman, Q., Rahman, M., Morshed, S., Islam, T., Sorif, S., Selim, M., Islam, M.R., and Hossain, M.M. (2015). Groundwater arsenic contamination in Bangladesh-21 Years of research. *J Trace Elem Med Biol* 31**,** 237-248.

Challenger, F. (1947). Biological methylation. *Sci Prog* 35**,** 396-416.
Chen, H.S., and Lipton, S.A. (2006). The chemical biology of clinically tolerated NMDA receptor antagonists. *J Neurochem* 97**,** 1611-1626.
Cho, Y., and Bannai, S. (1990). Uptake of glutamate and cysteine in C-6 glioma cells and in cultured astrocytes. *J Neurochem* 55**,** 2091-2097.
Cui, X., Kobayashi, Y., Hayakawa, T., and Hirano, S. (2004). Arsenic speciation in bile and urine following oral and intravenous exposure to inorganic and organic arsenics in rats. *Toxicol Sci* 82**,** 478-487.
Divakaruni, A.S., Wallace, M., Buren, C., Martyniuk, K., Andreyev, A.Y., Li, E., Fields, J.A., Cordes, T., Reynolds, I.J., Bloodgood, B.L., Raymond, L.A., Metallo, C.M., and Murphy, A.N. (2017). Inhibition of the mitochondrial pyruvate carrier protects from excitotoxic neuronal death. *J Cell Biol* 216**,** 1091-1105.
Dong, J., and Su, S.Y. (2009). The association between arsenic and children's intelligence: a meta-analysis. *Biol Trace Elem Res* 129**,** 88-93.
Drobna, Z., Styblo, M., and Thomas, D.J. (2009). An Overview of Arsenic Metabolism and Toxicity. *Curr Protoc Toxicol* 42**,** 4 31 31-34 31 36.
Duker, A.A., Carranza, E.J., and Hale, M. (2005). Arsenic geochemistry and health. *Environ Int* 31**,** 631-641.
Dunlop, J., Mcilvain, H.B., Carrick, T.A., Jow, B., Lu, Q., Kowal, D., Lin, S., Greenfield, A., Grosanu, C., Fan, K., Petroski, R., Williams, J., Foster, A., and Butera, J. (2005). Characterization of novel aryl-ether, biaryl, and fluorene aspartic acid and diaminopropionic acid analogs as potent inhibitors of the high-affinity glutamate transporter EAAT2. *Mol Pharmacol* 68**,** 974-982.
Dwivedi, N., and Flora, S.J. (2011). Concomitant exposure to arsenic and organophosphates on tissue oxidative stress in rats. *Food Chem Toxicol* 49**,** 1152-1159.
Ebert, D., Haller, R.G., and Walton, M.E. (2003). Energy contribution of octanoate to intact rat brain metabolism measured by 13C nuclear magnetic resonance spectroscopy. *J Neurosci* 23**,** 5928-5935.
Edmond, J., Robbins, R.A., Bergstrom, J.D., Cole, R.A., and De Vellis, J. (1987). Capacity for substrate utilization in oxidative metabolism by neurons, astrocytes, and oligodendrocytes from developing brain in primary culture. *J Neurosci Res* 18**,** 551-561.
Flora, S.J. (2011). Arsenic-induced oxidative stress and its reversibility. *Free Radic Biol Med* 51**,** 257-281.
Geng, A., Wang, X., Wu, L., Wang, F., Chen, Y., Yang, H., Zhang, Z., and Zhao, X. (2017). Arsenic accumulation and speciation in rice grown in arsanilic acid-elevated paddy soil. *Ecotoxicol Environ Saf* 137**,** 172-178.
Gray, L.R., Sultana, M.R., Rauckhorst, A.J., Oonthonpan, L., Tompkins, S.C., Sharma, A., Fu, X., Miao, R., Pewa, A.D., Brown, K.S., Lane, E.E., Dohlman, A., Zepeda-Orozco, D., Xie, J., Rutter, J., Norris, A.W., Cox, J.E., Burgess, S.C., Potthoff, M.J., and Taylor, E.B. (2015). Hepatic Mitochondrial Pyruvate Carrier 1 Is Required for Efficient Regulation of Gluconeogenesis and Whole-Body Glucose Homeostasis. *Cell Metab* 22**,** 669-681.
Halim, N.D., Mcfate, T., Mohyeldin, A., Okagaki, P., Korotchkina, L.G., Patel, M.S., Jeoung, N.H., Harris, R.A., Schell, M.J., and Verma, A. (2010). Phosphorylation status of pyruvate dehydrogenase distinguishes metabolic phenotypes of cultured rat brain astrocytes and neurons. *Glia* 58**,** 1168-1176.
Hamadani, J.D., Tofail, F., Nermell, B., Gardner, R., Shiraji, S., Bottai, M., Arifeen, S.E., Huda, S.N., and Vahter, M. (2011). Critical windows of exposure for arsenic-associated impairment of cognitive function in pre-school girls and boys: a population-based cohort study. *Int J Epidemiol* 40**,** 1593-1604.

Han, Y.H., Kim, S.H., Kim, S.Z., and Park, W.H. (2008). Apoptosis in arsenic trioxide-treated Calu-6 lung cells is correlated with the depletion of GSH levels rather than the changes of ROS levels. *J Cell Biochem* 104**,** 862-878.

Hayakawa, T., Kobayashi, Y., Cui, X., and Hirano, S. (2005). A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19. *Arch Toxicol* 79**,** 183-191.

Hirrlinger, J., and Dringen, R. (2005). Multidrug resistance protein 1-mediated export of glutathione and glutathione disulfide from brain astrocytes. *Methods Enzymol* 400**,** 395-409.

Hirrlinger, J., Schulz, J.B., and Dringen, R. (2002). Glutathione release from cultured brain cells: multidrug resistance protein 1 mediates the release of GSH from rat astroglial cells. *J Neurosci Res* 69**,** 318-326.

Hochwald, S.N., Harrison, L.E., Rose, D.M., Anderson, M., and Burt, M.E. (1996). gamma-Glutamyl transpeptidase mediation of tumor glutathione utilization in vivo. *J Natl Cancer Inst* 88**,** 193-197.

Hollensworth, S.B., Shen, C., Sim, J.E., Spitz, D.R., Wilson, G.L., and Ledoux, S.P. (2000). Glial cell type-specific responses to menadione-induced oxidative stress. *Free Radic Biol Med* 28**,** 1161-1174.

Hong, Y.S., Song, K.H., and Chung, J.Y. (2014). Health effects of chronic arsenic exposure. *J Prev Med Public Health* 47**,** 245-252.

Hosseini, M.J., Shaki, F., Ghazi-Khansari, M., and Pourahmad, J. (2013). Toxicity of Arsenic (III) on Isolated Liver Mitochondria: A New Mechanistic Approach. *Iran J Pharm Res* 12**,** 121-138.

Hu, Y., Su, L., and Snow, E.T. (1998). Arsenic toxicity is enzyme specific and its affects on ligation are not caused by the direct inhibition of DNA repair enzymes. *Mutat Res* 408**,** 203-218.

Jiang, S., Su, J., Yao, S., Zhang, Y., Cao, F., Wang, F., Wang, H., Li, J., and Xi, S. (2014). Fluoride and arsenic exposure impairs learning and memory and decreases mGluR5 expression in the hippocampus and cortex in rats. *PLoS One* 9**,** e96041.

Jin, Y., Sun, G., Li, X., Li, G., Lu, C., and Qu, L. (2004). Study on the toxic effects induced by different arsenicals in primary cultured rat astroglia. *Toxicol Appl Pharmacol* 196**,** 396-403.

Kala, S.V., Kala, G., Prater, C.I., Sartorelli, A.C., and Lieberman, M.W. (2004). Formation and urinary excretion of arsenic triglutathione and methylarsenic diglutathione. *Chem Res Toxicol* 17**,** 243-249.

King, M.P., and Attardi, G. (1989). Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* 246**,** 500-503.

Koehler, Y., Luther, E.M., Meyer, S., Schwerdtle, T., and Dringen, R. (2014). Uptake and toxicity of arsenite and arsenate in cultured brain astrocytes. *J Trace Elem Med Biol* 28**,** 328-337.

Kranich, O., Hamprecht, B., and Dringen, R. (1996). Different preferences in the utilization of amino acids for glutathione synthesis in cultured neurons and astroglial cells derived from rat brain. *Neurosci Lett* 219**,** 211-214.

Kruger, K., Straub, H., Hirner, A.V., Hippler, J., Binding, N., and Musshoff, U. (2009). Effects of monomethylarsonic and monomethylarsonous acid on evoked synaptic potentials in hippocampal slices of adult and young rats. *Toxicol Appl Pharmacol* 236**,** 115-123.

Kuehne, A., Emmert, H., Soehle, J., Winnefeld, M., Fischer, F., Wenck, H., Gallinat, S., Terstegen, L., Lucius, R., Hildebrand, J., and Zamboni, N. (2015). Acute Activation of

Oxidative Pentose Phosphate Pathway as First-Line Response to Oxidative Stress in Human Skin Cells. *Mol Cell* 59**,** 359-371.

Leslie, E.M., Haimeur, A., and Waalkes, M.P. (2004). Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required. *J Biol Chem* 279**,** 32700-32708.

Li, M., Jaffe, A.E., Straub, R.E., Tao, R., Shin, J.H., Wang, Y., Chen, Q., Li, C., Jia, Y., Ohi, K., Maher, B.J., Brandon, N.J., Cross, A., Chenoweth, J.G., Hoeppner, D.J., Wei, H., Hyde, T.M., Mckay, R., Kleinman, J.E., and Weinberger, D.R. (2016). A human-specific AS3MT isoform and BORCS7 are molecular risk factors in the 10q24.32 schizophrenia-associated locus. *Nat Med* 22**,** 649-656.

Lipton, S.A., and Rosenberg, P.A. (1994). Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med* 330**,** 613-622.

Liu, S.X., Davidson, M.M., Tang, X., Walker, W.F., Athar, M., Ivanov, V., and Hei, T.K. (2005). Mitochondrial damage mediates genotoxicity of arsenic in mammalian cells. *Cancer Res* 65**,** 3236-3242.

Liu, Z., Carbrey, J.M., Agre, P., and Rosen, B.P. (2004). Arsenic trioxide uptake by human and rat aquaglyceroporins. *Biochem Biophys Res Commun* 316**,** 1178-1185.

Liu, Z., Sanchez, M.A., Jiang, X., Boles, E., Landfear, S.M., and Rosen, B.P. (2006). Mammalian glucose permease GLUT1 facilitates transport of arsenic trioxide and methylarsonous acid. *Biochem Biophys Res Commun* 351**,** 424-430.

Lucarelli, G., Galleggiante, V., Rutigliano, M., Sanguedolce, F., Cagiano, S., Bufo, P., Lastilla, G., Maiorano, E., Ribatti, D., Giglio, A., Serino, G., Vavallo, A., Bettocchi, C., Selvaggi, F.P., Battaglia, M., and Ditonno, P. (2015). Metabolomic profile of glycolysis and the pentose phosphate pathway identifies the central role of glucose-6-phosphate dehydrogenase in clear cell-renal cell carcinoma. *Oncotarget* 6**,** 13371-13386.

Mark, L.P., Prost, R.W., Ulmer, J.L., Smith, M.M., Daniels, D.L., Strottmann, J.M., Brown, W.D., and Hacein-Bey, L. (2001). Pictorial review of glutamate excitotoxicity: fundamental concepts for neuroimaging. *AJNR Am J Neuroradiol* 22**,** 1813-1824.

Mathiisen, T.M., Lehre, K.P., Danbolt, N.C., and Ottersen, O.P. (2010). The perivascular astroglial sheath provides a complete covering of the brain microvessels: an electron microscopic 3D reconstruction. *Glia* 58**,** 1094-1103.

Mcbean, G.J. (2017). Cysteine, Glutathione, and Thiol Redox Balance in Astrocytes. *Antioxidants (Basel)* 6.

Mccommis, K.S., Chen, Z., Fu, X., Mcdonald, W.G., Colca, J.R., Kletzien, R.F., Burgess, S.C., and Finck, B.N. (2015). Loss of Mitochondrial Pyruvate Carrier 2 in the Liver Leads to Defects in Gluconeogenesis and Compensation via Pyruvate-Alanine Cycling. *Cell Metab* 22**,** 682-694.

Mckenna, M.C., Stridh, M.H., Mcnair, L.F., Sonnewald, U., Waagepetersen, H.S., and Schousboe, A. (2016). Glutamate oxidation in astrocytes: Roles of glutamate dehydrogenase and aminotransferases. *J Neurosci Res* 94**,** 1561-1571.

Mckenna, M.C., Tildon, J.T., Stevenson, J.H., Boatright, R., and Huang, S. (1993). Regulation of energy metabolism in synaptic terminals and cultured rat brain astrocytes: differences revealed using aminooxyacetate. *Dev Neurosci* 15**,** 320-329.

Mckenna, M.C., Tildon, J.T., Stevenson, J.H., Huang, X., and Kingwell, K.G. (1995). Regulation of mitochondrial and cytosolic malic enzymes from cultured rat brain astrocytes. *Neurochem Res* 20**,** 1491-1501.

Mckenna, M.C., Waagepetersen, H.S., Schousboe, A., and Sonnewald, U. (2006). Neuronal and astrocytic shuttle mechanisms for cytosolic-mitochondrial transfer of reducing

equivalents: current evidence and pharmacological tools. *Biochem Pharmacol* 71**,** 399-407.

Meyer, N., Koehler, Y., Tulpule, K., and Dringen, R. (2013). Arsenate accumulation and arsenate-induced glutathione export in astrocyte-rich primary cultures. *Neurochem Int* 62**,** 1012-1019.

Nemeti, B., and Gregus, Z. (2002). Mitochondria work as reactors in reducing arsenate to arsenite. *Toxicol Appl Pharmacol* 182**,** 208-218.

Nordstrom, D.K. (2002). Public health. Worldwide occurrences of arsenic in ground water. *Science* 296**,** 2143-2145.

Pardo, B., Rodrigues, T.B., Contreras, L., Garzon, M., Llorente-Folch, I., Kobayashi, K., Saheki, T., Cerdan, S., and Satrustegui, J. (2011). Brain glutamine synthesis requires neuronal-born aspartate as amino donor for glial glutamate formation. *J Cereb Blood Flow Metab* 31**,** 90-101.

Partridge, M.A., Huang, S.X., Hernandez-Rosa, E., Davidson, M.M., and Hei, T.K. (2007). Arsenic induced mitochondrial DNA damage and altered mitochondrial oxidative function: implications for genotoxic mechanisms in mammalian cells. *Cancer Res* 67**,** 5239-5247.

Piao, F., Ma, N., Hiraku, Y., Murata, M., Oikawa, S., Cheng, F., Zhong, L., Yamauchi, T., Kawanishi, S., and Yokoyama, K. (2005). Oxidative DNA damage in relation to neurotoxicity in the brain of mice exposed to arsenic at environmentally relevant levels. *J Occup Health* 47**,** 445-449.

Pichili, V.B., Rao, K.V., Jayakumar, A.R., and Norenberg, M.D. (2007). Inhibition of glutamine transport into mitochondria protects astrocytes from ammonia toxicity. *Glia* 55**,** 801-809.

Pompella, A., De Tata, V., Paolicchi, A., and Zunino, F. (2006). Expression of gamma-glutamyltransferase in cancer cells and its significance in drug resistance. *Biochem Pharmacol* 71**,** 231-238.

Prakash, C., and Kumar, V. (2016). Arsenic-induced mitochondrial oxidative damage is mediated by decreased PGC-1alpha expression and its downstream targets in rat brain. *Chem Biol Interact* 256**,** 228-235.

Prakash, C., Soni, M., and Kumar, V. (2015). Biochemical and Molecular Alterations Following Arsenic-Induced Oxidative Stress and Mitochondrial Dysfunction in Rat Brain. *Biol Trace Elem Res* 167**,** 121-129.

Rajpert-De Meyts, E., Shi, M., Chang, M., Robison, T.W., Groffen, J., Heisterkamp, N., and Forman, H.J. (1992). Transfection with gamma-glutamyl transpeptidase enhances recovery from glutathione depletion using extracellular glutathione. *Toxicol Appl Pharmacol* 114**,** 56-62.

Ramanathan, K., Shila, S., Kumaran, S., and Panneerselvam, C. (2003). Ascorbic acid and alpha-tocopherol as potent modulators on arsenic induced toxicity in mitochondria. *J Nutr Biochem* 14**,** 416-420.

Ramos-Chavez, L.A., Rendon-Lopez, C.R., Zepeda, A., Silva-Adaya, D., Del Razo, L.M., and Gonsebatt, M.E. (2015). Neurological effects of inorganic arsenic exposure: altered cysteine/glutamate transport, NMDA expression and spatial memory impairment. *Front Cell Neurosci* 9**,** 21.

Ramsay, E.E., and Dilda, P.J. (2014). Glutathione S-conjugates as prodrugs to target drug-resistant tumors. *Front Pharmacol* 5**,** 181.

Rathinam, M.L., Watts, L.T., Narasimhan, M., Riar, A.K., Mahimainathan, L., and Henderson, G.I. (2012). Astrocyte mediated protection of fetal cerebral cortical neurons from rotenone and paraquat. *Environ Toxicol Pharmacol* 33**,** 353-360.

Roberts, R.C., Roche, J.K., and Mccullumsmith, R.E. (2014). Localization of excitatory amino acid transporters EAAT1 and EAAT2 in human postmortem cortex: a light and electron microscopic study. *Neuroscience* 277**,** 522-540.

Rodriguez, V.M., Del Razo, L.M., Limon-Pacheco, J.H., Giordano, M., Sanchez-Pena, L.C., Uribe-Querol, E., Gutierrez-Ospina, G., and Gonsebatt, M.E. (2005). Glutathione reductase inhibition and methylated arsenic distribution in Cd1 mice brain and liver. *Toxicol Sci* 84**,** 157-166.

Rosado, J.L., Ronquillo, D., Kordas, K., Rojas, O., Alatorre, J., Lopez, P., Garcia-Vargas, G., Del Carmen Caamano, M., Cebrian, M.E., and Stoltzfus, R.J. (2007). Arsenic exposure and cognitive performance in Mexican schoolchildren. *Environ Health Perspect* 115**,** 1371-1375.

Sagara, J., Makino, N., and Bannai, S. (1996). Glutathione efflux from cultured astrocytes. *J Neurochem* 66**,** 1876-1881.

Seib, T.M., Patel, S.A., and Bridges, R.J. (2011). Regulation of the system x(C)-cystine/glutamate exchanger by intracellular glutathione levels in rat astrocyte primary cultures. *Glia* 59**,** 1387-1401.

Shukalek, C.B., Swanlund, D.P., Rousseau, R.K., Weigl, K.E., Marensi, V., Cole, S.P., and Leslie, E.M. (2016). Arsenic Triglutathione [As(GS)3] Transport by Multidrug Resistance Protein 1 (MRP1/ABCC1) Is Selectively Modified by Phosphorylation of Tyr920/Ser921 and Glycosylation of Asn19/Asn23. *Mol Pharmacol* 90**,** 127-139.

Smith, N.M., Lee, R., Heitkemper, D.T., Denicola Cafferky, K., Haque, A., and Henderson, A.K. (2006). Inorganic arsenic in cooked rice and vegetables from Bangladeshi households. *Sci Total Environ* 370**,** 294-301.

Sonnewald, U., Westergaard, N., and Schousboe, A. (1997). Glutamate transport and metabolism in astrocytes. *Glia* 21**,** 56-63.

Sullivan, L.B., Gui, D.Y., Hosios, A.M., Bush, L.N., Freinkman, E., and Vander Heiden, M.G. (2015). Supporting Aspartate Biosynthesis Is an Essential Function of Respiration in Proliferating Cells. *Cell* 162**,** 552-563.

Supplie, L.M., Duking, T., Campbell, G., Diaz, F., Moraes, C.T., Gotz, M., Hamprecht, B., Boretius, S., Mahad, D., and Nave, K.A. (2017). Respiration-Deficient Astrocytes Survive As Glycolytic Cells In Vivo. *J Neurosci* 37**,** 4231-4242.

Szatkowski, M., Barbour, B., and Attwell, D. (1990). Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* 348**,** 443-446.

Tadepalle, N., Koehler, Y., Brandmann, M., Meyer, N., and Dringen, R. (2014). Arsenite stimulates glutathione export and glycolytic flux in viable primary rat brain astrocytes. *Neurochem Int* 76**,** 1-11.

Vacanti, N.M., Divakaruni, A.S., Green, C.R., Parker, S.J., Henry, R.R., Ciaraldi, T.P., Murphy, A.N., and Metallo, C.M. (2014). Regulation of substrate utilization by the mitochondrial pyruvate carrier. *Mol Cell* 56**,** 425-435.

Villa-Bellosta, R., and Sorribas, V. (2010). Arsenate transport by sodium/phosphate cotransporter type IIb. *Toxicol Appl Pharmacol* 247**,** 36-40.

Vogel, R., Hamprecht, B., and Wiesinger, H. (1998). Malic enzyme isoforms in astrocytes: comparative study on activities in rat brain tissue and astroglia-rich primary cultures. *Neurosci Lett* 247**,** 123-126.

Waagepetersen, H.S., Qu, H., Hertz, L., Sonnewald, U., and Schousboe, A. (2002). Demonstration of pyruvate recycling in primary cultures of neocortical astrocytes but not in neurons. *Neurochem Res* 27**,** 1431-1437.

Waagepetersen, H.S., Qu, H., Schousboe, A., and Sonnewald, U. (2001). Elucidation of the quantitative significance of pyruvate carboxylation in cultured cerebellar neurons and astrocytes. *J Neurosci Res* 66**,** 763-770.

Wang, X.F., and Cynader, M.S. (2000). Astrocytes provide cysteine to neurons by releasing glutathione. *J Neurochem* 74**,** 1434-1442.

Wang, Y., Zhao, F., Liao, Y., Jin, Y., and Sun, G. (2012). Arsenic exposure and glutamate-induced gliotransmitter release from astrocytes. *Neural Regen Res* 7**,** 2439-2445.

Warburg, O. (1956). On the origin of cancer cells. *Science* 123**,** 309-314.

Wasserman, G.A., Liu, X., Loiacono, N.J., Kline, J., Factor-Litvak, P., Van Geen, A., Mey, J.L., Levy, D., Abramson, R., Schwartz, A., and Graziano, J.H. (2014). A cross-sectional study of well water arsenic and child IQ in Maine schoolchildren. *Environ Health* 13**,** 23.

Wasserman, G.A., Liu, X., Parvez, F., Ahsan, H., Factor-Litvak, P., Van Geen, A., Slavkovich, V., Loiacono, N.J., Cheng, Z., Hussain, I., Momotaj, H., and Graziano, J.H. (2004). Water arsenic exposure and children's intellectual function in Araihazar, Bangladesh. *Environ Health Perspect* 112**,** 1329-1333.

Waters, S.B., Devesa, V., Del Razo, L.M., Styblo, M., and Thomas, D.J. (2004). Endogenous reductants support the catalytic function of recombinant rat cyt19, an arsenic methyltransferase. *Chem Res Toxicol* 17**,** 404-409.

Weightman Potter, P.G., Vlachaki Walker, J.M., Robb, J.L., Chilton, J.K., Williamson, R., Randall, A.D., Ellacott, K.L.J., and Beall, C. (2019). Basal fatty acid oxidation increases after recurrent low glucose in human primary astrocytes. *Diabetologia* 62**,** 187-198.

Westergaard, N., Drejer, J., Schousboe, A., and Sonnewald, U. (1996). Evaluation of the importance of transamination versus deamination in astrocytic metabolism of [U-13C]glutamate. *Glia* 17**,** 160-168.

Yadav, R.S., Shukla, R.K., Sankhwar, M.L., Patel, D.K., Ansari, R.W., Pant, A.B., Islam, F., and Khanna, V.K. (2010). Neuroprotective effect of curcumin in arsenic-induced neurotoxicity in rats. *Neurotoxicology* 31**,** 533-539.

Yang, C., Ko, B., Hensley, C.T., Jiang, L., Wasti, A.T., Kim, J., Sudderth, J., Calvaruso, M.A., Lumata, L., Mitsche, M., Rutter, J., Merritt, M.E., and Deberardinis, R.J. (2014). Glutamine oxidation maintains the TCA cycle and cell survival during impaired mitochondrial pyruvate transport. *Mol Cell* 56**,** 414-424.

Zhang, Y., Zhang, X., and Qu, S. (2015). Ceftriaxone Protects Astrocytes from MPP(+) via Suppression of NF-kappaB/JNK/c-Jun Signaling. *Mol Neurobiol* 52**,** 78-92.

Zhao, F., Liao, Y., Jin, Y., Li, G., Lv, X., and Sun, G. (2012). Effects of arsenite on glutamate metabolism in primary cultured astrocytes. *Toxicol In Vitro* 26**,** 24-31.

CHAPTER 5
CONCLUSIONS AND FUTURE EXPERIMENTS

Chapters 2 and 3 provide in depth explorations of current literature highlighting the complexity and interconnectedness of the cells of the central nervous system, and the specialized metabolic functions of astrocytes, with an emphasis on mitochondrial function. In chapter 4, experiments were performed to explore the alteration of astrocyte metabolism when challenged with the xenobiotic arsenic. The results of these experiments show that astrocytes were shown to be dependent upon *de novo* glutathione (GSH) synthesis during arsenic exposure. A time course of intracellular arsenic accumulation at various arsenic concentrations illustrated that there was an acute phase accumulation of arsenic in astrocytes, followed by an adaptive chronic phase where arsenic accumulation was halted or reversed at sub-lethal sodium arsenite concentrations. Further, inhibition of the multidrug resistance protein 1 (MRP1) led to an accumulation of intracellular arsenic in astrocytes and sensitized astrocytes to arsenic. Arsenic exposure led to an accumulation of glutamate in the media of astrocyte cultures, an effect exacerbated by the inhibition of excitatory amino acid transporters 1 and 2 (EAAT1/2). Additionally, application of mitochondrial toxins or inhibition of carbon inputs to the mitochondria or transamination processes sensitized astrocytes to arsenic. The relevance of each of these results is briefly covered below.

Arsenic exposure requires de novo synthesis of GSH to maintain astrocyte viability, illustrating that the reduction of existing oxidized glutathione (GSSG) pools is insufficient to maintain astrocyte viability and REDOX homeostasis in the presence of arsenic. Cellular and DNA damage from reactive oxygen species (ROS) has been observed during arsenic exposure in other cell types (Hei et al., 1998;Liu et al., 2001). While low dose arsenic does not generate detectable ROS in viable astrocytes (Catanzaro et al., 2010), it should be noted that astrocyte intracellular GSH content does not significantly change at the same low doses after 24hrs (Zhao

et al., 2012). This thesis reinforces that arsenic induced ROS is not a significant factor by illustrating that 6-aminonicotinamide (6-AN), an inhibitor of the pentose phosphate pathway (PPP), has no significant effect on arsenic sensitization in astrocytes, while inhibition of GSH synthesis by L-buthionine-sulfoximine (BSO) profoundly sensitized the astrocytes to arsenic.

The generation of a time course of intracellular arsenic accumulation at low doses was important to illustrate that the detoxification mechanisms in astrocytes exposed to arsenic are not immediate, and that 10 μM sodium arsenite is near the limit of the detoxification capacity of astrocytes. The time-course generated in this thesis illustrates that within the first three hours of exposure, intracellular arsenic accumulation increases, followed by a reversal (5 μM sodium arsenite) or cessation (10 μM sodium arsenite) of arsenic accumulation beyond that point at sub-lethal concentrations. The time course of 20 μM sodium arsenite does not clearly demonstrate such a change, and the astrocytes show cell death at 48hrs at this concentration. Prior literature illustrates that arsenic accumulation measurements in astrocytes has used either very high arsenic concentrations (Koehler and Dringen, 2013;Koehler et al., 2014), and/or have used very short exposure times (Koehler and Dringen, 2013;Tadepalle et al., 2014). The time course created in this thesis demonstrates that cellular responses to xenobiotics clearly differ significantly over the exposure time (in hours for astrocytes and arsenic), and that metabolic changes due to xenobiotic exposure may be masked if assays are performed too quickly following exposure. Further, this the near steady state of intracellular arsenic accumulation in astrocytes at 10 μM sodium arsenite provide an experimental condition whereby disruptions to detoxification processes may be readily observed in terms of viability.

The results obtained from the inhibition of MRP1 provide further evidence in the contended role of this transporter in arsenic exposure. MRP1 has been described to efflux a glutathionylated arsenical complex (Leslie et al., 2004;Carew et al., 2011) (reviewed in (Leslie,

2012)), or to co-transport arsenic and GSH out of the cell (Salerno et al., 2002). MRP1 is also responsible for the neuroprotective export of GSH by astrocytes to neurons, reviewed in detail in Chapters 2 and 3. Studies into the effects of MRP1 inhibition by MK571 during arsenic exposure in astrocytes found that arsenic accumulation increased in the presence of MK571 but no effect on astrocyte viability was observed (Meyer et al., 2013;Tadepalle et al., 2014). A separate study found similar increases in arsenic accumulation with MK571, but observed cytotoxic effects as well after 72hrs (Gulden et al., 2017). Our data reinforce that MRP1 inhibition leads to an increase in intracellular arsenic accumulation and compromises cell viability.

During arsenic exposure, excitotoxic concentrations of glutamate accumulate in the media of astrocyte cultures. As detailed in chapter 4, NMR results show that glucose carbons are being metabolized toward the production of glutamate that principally is found in the media, rather than within the astrocytes. Further NMR experiments are underway to better isolate the precise metabolic pathways utilized for this glutamate production but were not ready at the time of this thesis. A previous report that found an approximate 1.5 μM increase in extracellular glutamate after 24hrs exposure of 10 μM sodium arsenite (Wang et al., 2012). An enzymatic assay was performed (see chapter 4) and the extracellular glutamate observed here was consistently above 30 μM. Glutamate is an excitatory neurotransmitter, and neuronal excitotoxicity has been observed in the 2-5 μM glutamate range (Lipton and Rosenberg, 1994;Mark et al., 2001;Chen and Lipton, 2006). This finding has profound implications for neuronal survival when astrocytes are exposed to arsenic. An additional quantification technique beyond the enzymatic assay performed is warranted, given the similarity of this thesis's methodology and that described above (Wang et al., 2012). Further, it is important to discover how this glutamate is released, as the mechanism may lie in a region of the astrocyte far from a synaptic cleft (see chapter 3 for more detail). Regardless, this is a novel finding.

This thesis begins to explore two possible mechanisms for the release of glutamate from astrocytes. The first is by the cystine-glutamate exchanger (xCT), which effluxes glutamate to take in cystine, a rate limiting component of GSH synthesis. Viability experiments utilizing sulfasalazine (SAS), an inhibitor of xCT, showed a minor but significant reduction in astrocyte viability. Experiments are planned to test if SAS significantly impacts the accumulation of extracellular glutamate but were not completed at the time of this thesis. The second is by reversal of EAAT1 and EAAT2 (Szatkowski et al., 1990), transporters that normally are involved in the uptake of glutamate (see chapter 3 and 4). Inhibition of both EAAT1/2 significantly increased the extracellular glutamate concentration, including in the absence of arsenic. This data indicates that EAAT1/2 are not only not the source of glutamate release, but are also active to counteract some other mechanism of glutamate release that occurs during culture conditions, which requires further characterization. A test of extracellular glutamate quantification with EAAT1 and EAAT2 independently inhibited is planned for future tests. Viability tests of EAAT1 and EAAT2 inhibition demonstrated that EAAT1 inhibition, but not EAAT2, sensitized astrocytes to arsenic, indicating that EAAT1 is playing a vital function during toxicity. One possibility is that intracellular glutamate is being depleted and must be synthesized from glucose and/or reuptaken by EAAT1 in order to maintain GSH synthesis.

Data from this thesis demonstrates that mitochondrial metabolism is required in astrocytes during arsenic exposure. As outlined in more detail in chapters 2-4, astrocytes are principally glycolytic, do not require oxidative phosphorylation to perform their functions, and are resistant to mitochondrial toxins (Bolanos et al., 1995;Almeida et al., 2001;Supplie et al., 2017). This thesis confirmed that astrocytes remained viable in the presence of mitochondrial toxins, but these toxins sensitized the astrocytes to arsenic, indicating that the mitochondria are performing a vital function during arsenic exposure. The prior data from this thesis indicates that

it may be tied to glutamate synthesis from glucose (NMR results) to synthesize GSH (BSO results) and/or to compensate for the glutamate effluxed during arsenic exposure (NMR and Glutamate-Glo assay). To this end, inhibition of mitochondrial carbon inputs by UK5099, an inhibitor pyruvate entry to the mitochondria, and by etomoxir, an inhibitor of medium and long chain fatty acid entry to the mitochondria. Similarly, inhibition of transamination, a component of glutamate synthesis, by aminooxyacetic acid (AOAA) was tested. All three inhibitors (UK5099, etomoxir, AOAA) significantly decreased astrocyte viability in the presence of arsenic. The AOAA result indicates that a transamination process is involved in arsenic detoxification, and warrants further exploration, as transamination is not limited to mitochondrial processes.

Fatty acid utilization as a mitochondrial carbon source in astrocytes is a recent subject of research (see chapter 3), and the etomoxir results reinforce this possibility. To further characterize this, a Mitochondrial Stress Test (Agilent) was performed in the presence of etomoxir and arsenic. The co-administration of etomoxir and arsenic seems to cause major mitochondrial dysfunction with a drastically reduced mitochondrial capacity for OXPHOS. This could be a sign of mitochondrial degradation at this time point, and requires further exploration to determine the cause. It should be noted that a 200 μM etomoxir treatment may have off target effects, since etomoxir has been proposed to inhibit glutamine metabolism (O'Connor et al., 2018), to inhibit complex I activity (Yao et al., 2018), and to deplete the cell of CoA (Divakaruni et al., 2018). While the minimal basal activity of etomoxir may be due to complex I inhibition, the effect of etomoxir on maximal respiration was minimal. To test this, the Mitochondrial Stress Test should be repeated with just rotenone, or another complex I inhibitor, to see if the same effect was observed. Additionally, the test should also be performed with UK5099 and AOAA.

What is clear is that a complex process occurs in astrocytes to facilitate survival in the presence of the xenobiotic arsenic. This survival facilitation occurs in a time dependent manner

that was not clear from prior literature results, and involves both the synthesis of GSH as well as glutamate. Consequently, it appears that in adapting to survive in the presence of arsenic, astrocytes may produce a toxic environment for nearby neurons. While many facets of the metabolic response remain to be explored and characterized, it is clear that astrocytes depend upon the biosynthetic properties of mitochondria to survive.

5.1 References

Almeida, A., Almeida, J., Bolanos, J.P., and Moncada, S. (2001). Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. *Proc Natl Acad Sci U S A* 98**,** 15294-15299.

Bolanos, J.P., Heales, S.J., Land, J.M., and Clark, J.B. (1995). Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurones and astrocytes in primary culture. *J Neurochem* 64**,** 1965-1972.

Carew, M.W., Naranmandura, H., Shukalek, C.B., Le, X.C., and Leslie, E.M. (2011). Monomethylarsenic diglutathione transport by the human multidrug resistance protein 1 (MRP1/ABCC1). *Drug Metab Dispos* 39**,** 2298-2304.

Catanzaro, I., Schiera, G., Sciandrello, G., Barbata, G., Caradonna, F., Proia, P., and Di Liegro, I. (2010). Biological effects of inorganic arsenic on primary cultures of rat astrocytes. *Int J Mol Med* 26**,** 457-462.

Chen, H.S., and Lipton, S.A. (2006). The chemical biology of clinically tolerated NMDA receptor antagonists. *J Neurochem* 97**,** 1611-1626.

Divakaruni, A.S., Hsieh, W.Y., Minarrieta, L., Duong, T.N., Kim, K.K.O., Desousa, B.R., Andreyev, A.Y., Bowman, C.E., Caradonna, K., Dranka, B.P., Ferrick, D.A., Liesa, M., Stiles, L., Rogers, G.W., Braas, D., Ciaraldi, T.P., Wolfgang, M.J., Sparwasser, T., Berod, L., Bensinger, S.J., and Murphy, A.N. (2018). Etomoxir Inhibits Macrophage Polarization by Disrupting CoA Homeostasis. *Cell Metab* 28**,** 490-503 e497.

Gulden, M., Appel, D., Syska, M., Uecker, S., Wages, F., and Seibert, H. (2017). Chrysin and silibinin sensitize human glioblastoma cells for arsenic trioxide. *Food Chem Toxicol* 105**,** 486-497.

Hei, T.K., Liu, S.X., and Waldren, C. (1998). Mutagenicity of arsenic in mammalian cells: role of reactive oxygen species. *Proc Natl Acad Sci U S A* 95**,** 8103-8107.

Koehler, Y., and Dringen, R. (2013). Characterization of arsenate uptake by cultured primary rat astrocytes. *Neurochem Res* 38**,** 1785-1790.

Koehler, Y., Luther, E.M., Meyer, S., Schwerdtle, T., and Dringen, R. (2014). Uptake and toxicity of arsenite and arsenate in cultured brain astrocytes. *J Trace Elem Med Biol* 28**,** 328-337.

Leslie, E.M. (2012). Arsenic-glutathione conjugate transport by the human multidrug resistance proteins (MRPs/ABCCs). *J Inorg Biochem* 108**,** 141-149.

Leslie, E.M., Haimeur, A., and Waalkes, M.P. (2004). Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required. *J Biol Chem* 279**,** 32700-32708.

Lipton, S.A., and Rosenberg, P.A. (1994). Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med* 330**,** 613-622.

Liu, S.X., Athar, M., Lippai, I., Waldren, C., and Hei, T.K. (2001). Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. *Proc Natl Acad Sci U S A* 98**,** 1643-1648.

Mark, L.P., Prost, R.W., Ulmer, J.L., Smith, M.M., Daniels, D.L., Strottmann, J.M., Brown, W.D., and Hacein-Bey, L. (2001). Pictorial review of glutamate excitotoxicity: fundamental concepts for neuroimaging. *AJNR Am J Neuroradiol* 22**,** 1813-1824.

Meyer, N., Koehler, Y., Tulpule, K., and Dringen, R. (2013). Arsenate accumulation and arsenate-induced glutathione export in astrocyte-rich primary cultures. *Neurochem Int* 62**,** 1012-1019.

O'connor, R.S., Guo, L., Ghassemi, S., Snyder, N.W., Worth, A.J., Weng, L., Kam, Y., Philipson, B., Trefely, S., Nunez-Cruz, S., Blair, I.A., June, C.H., and Milone, M.C. (2018). The CPT1a inhibitor, etomoxir induces severe oxidative stress at commonly used concentrations. *Sci Rep* 8**,** 6289.

Salerno, M., Petroutsa, M., and Garnier-Suillerot, A. (2002). The MRP1-mediated effluxes of arsenic and antimony do not require arsenic-glutathione and antimony-glutathione complex formation. *J Bioenerg Biomembr* 34**,** 135-145.

Supplie, L.M., Duking, T., Campbell, G., Diaz, F., Moraes, C.T., Gotz, M., Hamprecht, B., Boretius, S., Mahad, D., and Nave, K.A. (2017). Respiration-Deficient Astrocytes Survive As Glycolytic Cells In Vivo. *J Neurosci* 37**,** 4231-4242.

Szatkowski, M., Barbour, B., and Attwell, D. (1990). Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* 348**,** 443-446.

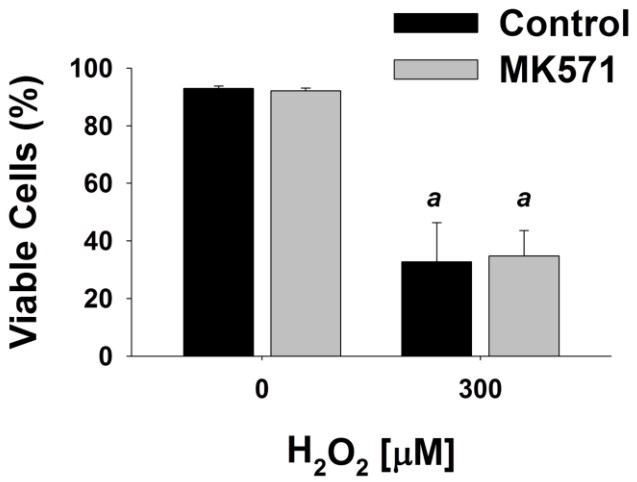
Tadepalle, N., Koehler, Y., Brandmann, M., Meyer, N., and Dringen, R. (2014). Arsenite stimulates glutathione export and glycolytic flux in viable primary rat brain astrocytes. *Neurochem Int* 76**,** 1-11.

Wang, Y., Zhao, F., Liao, Y., Jin, Y., and Sun, G. (2012). Arsenic exposure and glutamate-induced gliotransmitter release from astrocytes. *Neural Regen Res* 7**,** 2439-2445.

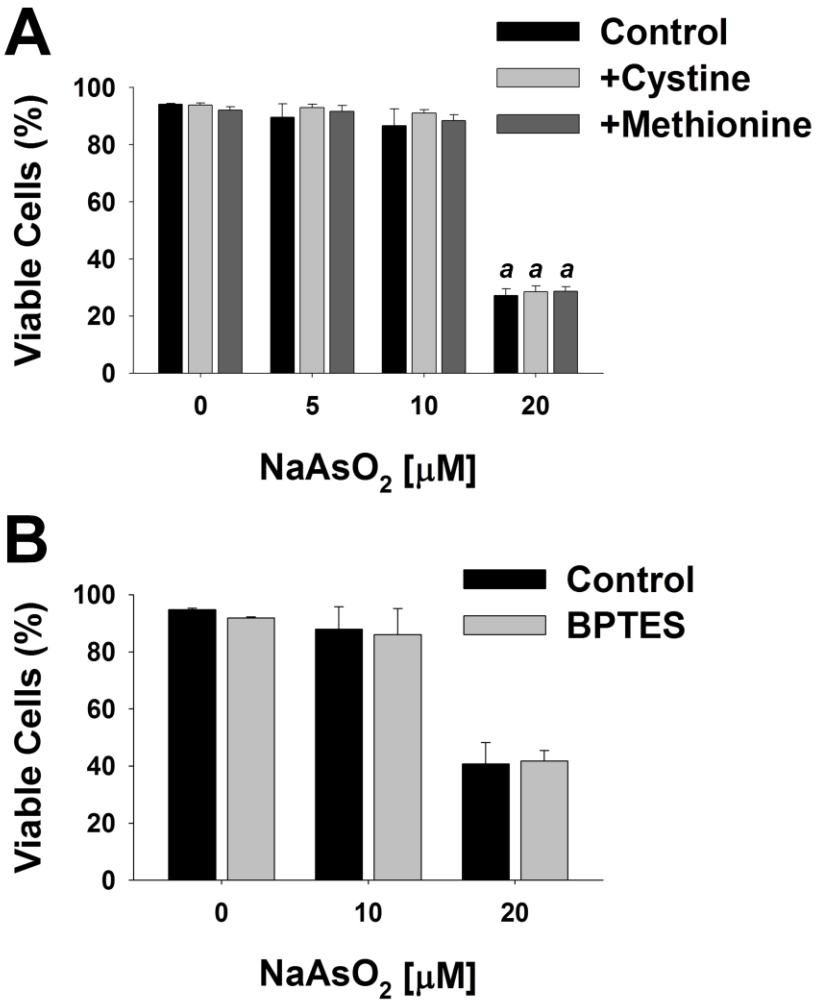
Yao, C.H., Liu, G.Y., Wang, R., Moon, S.H., Gross, R.W., and Patti, G.J. (2018). Identifying off-target effects of etomoxir reveals that carnitine palmitoyltransferase I is essential for cancer cell proliferation independent of beta-oxidation. *PLoS Biol* 16**,** e2003782.

Zhao, F., Liao, Y., Jin, Y., Li, G., Lv, X., and Sun, G. (2012). Effects of arsenite on glutamate metabolism in primary cultured astrocytes. *Toxicol In Vitro* 26**,** 24-31.

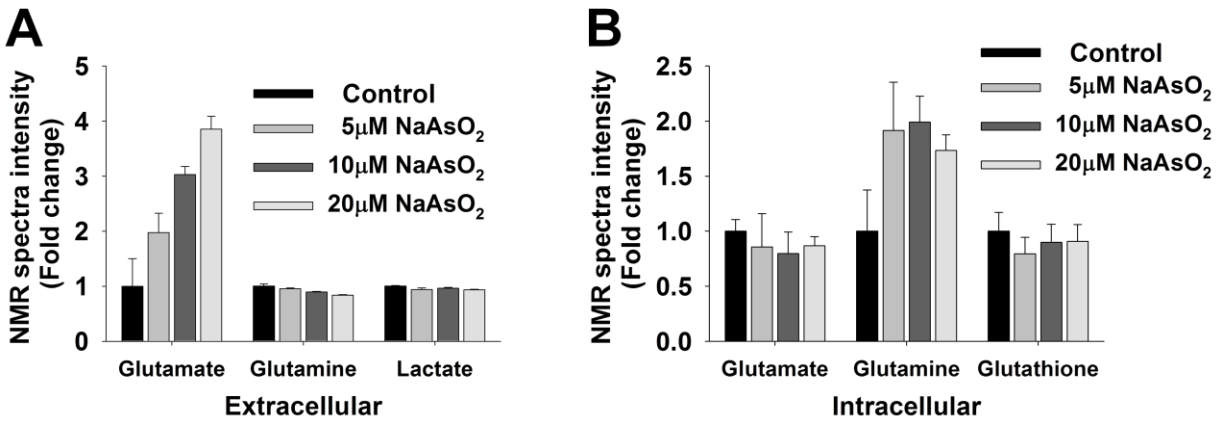
APPENDIX A: Supplemental Figures



Appendix Figure 1: Viable cell population determined by flow cytometry following 48hrs of hydrogen peroxide vs. 25μM MK571. ***a,b***: p<.05 Two Way ANOVA vs. the untreated control (*a*) or the control at the same arsenic concentration (*b*).



Appendix Figure 2: (A-B) Viable cell population determined by flow cytometry following 48hrs of the indicated arsenic vs. (A) 200μM cystine, 200μM methionine, or (B) 2μM BPTES. ***a***: $p<.05$ Two Way ANOVA vs. the untreated control (*a*). Note: BPTES results are preliminary, and only have 2 replicates.



Appendix Figure 3: Preliminary 2D-NMR (^{1}H-^{13}C) metabolomics performed on astrocytes treated with the indicated arsenic concentrations in DMEM containing 100μM U-^{13}C/^{15}N-glutamate for 24hrs. Extracellular **(A)** and intracellular **(B)** components were measured for each sample and represented as fold change compared to control.